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Hertwig's epithelial root sheath, enamel matrix proteins, and initiation of cementogenesis in porcine teeth

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Abstract

Objectives: The aim of this study was to analyze the association between Hertwig's epithelial root sheath (HERS) cells, enamel matrix proteins (EMPs), and cementogenesis.

Material and Methods: Porcine teeth were examined at the beginning of root formation by light and transmission electron microscopy. Colloidal gold immunocytochemistry was used to analyze the protein expression of amelogenin and ameloblastin.

Results: Before and during disintegration of HERS, its cells displayed the cytologic features of protein synthesis and secretion. While some cells assumed an ameloblast-like phenotype, others extended their territory away from the root surface. A collagenous matrix filled the widening intercellular spaces, and tonofilaments and desmosomes were still present in cells featuring the morphologic characteristics of cementoblasts. Labeling for amelogenin was observed but ameloblastin was not immunodetected. Labeling was associated with organic matrix deposits that were sporadically and randomly distributed both along the root surface and away from it among the dissipated epithelial cells.

Conclusions: These findings suggest that HERS' cells occasionally assume a lingering ameloblastic activity at the beginning of root formation in the pig. While the results do not support the hypothesis of a causal relationship between EMPs and cementogenesis, they lend support to the concept of an epithelial origin of cementoblasts.

Key words: cementoblast; cementogenesis; enamel matrix proteins; epithelial–mesenchymal transformation; pig; Hertwig's epithelial root sheath

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The regeneration of periodontal tissues destroyed or lost due to periodontitis is a major objective in periodontal therapy. A derivative of enamel matrix proteins (EMPs) extracted from porcine tooth buds has recently been introduced as a device to achieve this goal predictably. The rationale for the use of this protein-based, commercial product is the assumption that EMPs, synthesized and secreted by cells of the Hertwig's epithelial root sheath (HERS), induce the differentiation of dental follicle cells into cementoblasts (Hammarström 1997). In particular, amelogenins are believed to initiate specifically the genesis of acellular extrinsic fiber cementum (Hammarström 1997, Hammarström et al. 1997). While experimental data confirming this assumption are lacking, several studies used biochemical (Slavkin et al. 1989a, b, Fukae et al. 2001) and histological (Slavkin et al. 1989b, Luo et al. 1991, Bosshardt & Schroeder 1996, Fong et al. 1996, Thomas et al. 1997, Bosshardt & Nanci 1998, 2000, Fong & Hammarström 2000, Hu et al. 2001) techniques to demonstrate protein and gene expression of EMPs during root development. Slavkin et al. (1989b) demonstrated both amelogenin and enamelin protein expression along the cervical-most portion of growing molars from young mice, a region where acellular extrinsic fiber cementum prevails. Others (Luo et al. 1991, Fong et al. 1996, Hu et al. 2001) were, however, not able to detect amelogenin mRNA in cells lining forming roots. This discrepancy raised the

possibility that EMPs other than amelogenins are expressed during root development. Amelin (Cerny et al. 1996) and ameloblastin (Krebsbach et al. 1996), two novel ameloblast genes, believed to encode for the same protein, were detected in 1996 by two independent research groups. Amelin (Fong et al. 1996) and ameloblastin (Thomas et al. 1997) mRNAs were detected in cells lining the middle and apical root portions of rat molars, thus in regions not associated with the formation of acellular extrinsic fiber cementum. Further analyses of protein and/or gene expression of ameloblastin/amelin in rat molars confirmed its occasional association with cellular intrinsic fiber cementum (Bosshardt & Nanci 2000, Fong & Hammarström 2000). From another immunocytochemical study, it was concluded that the cervical root portion labeled for ameloblastin and amelogenin, because the crown enamel matrix extends for a short stretch over root dentin (Bosshardt & Nanci 1998). Thus, the above studies do not demonstrate a consistent pattern of EMP expression during root development. It is, therefore, difficult to reconcile these observations with the proposed role of EMPs in the induction of cementogenesis.

Very recently, Fukae et al. (2001) detected small amounts of amelogenin, enamelin, and sheathlin (another term for amelin/ameloblastin) in samples from the apical portion of the forming root in porcine teeth by immunoblot analysis and reverse-transcriptase polymerase chain reaction. These techniques could, however, neither demonstrate whether the EMPs were located in the dentin matrix, the dentin–cementum interface, or the cementum matrix proper nor specify their cellular origin. Since pre-/odontoblasts were shown to express EMPs (Bègue-Kirn et al. 1998, Fong et al. 1998, Veis et al. 2000, Oida et al. 2002), Fukae et al. (2001) could also at best only speculate on HERS' involvement in the production of EMPs and its putative function(s) in root development. Because of the uncertain role of HERS in both EMP synthesis and the initiation of cementogenesis, the aims of this study were to (a) analyze the morphological changes HERS' cells undergo at the onset of cementum matrix deposition and (b) immunolocalize amelogenin and ameloblastin along the roots of porcine teeth during early stages of root development.

Material and Methods

Tissue processing for ultrastructural and immunocytochemical analyses

Teeth from two domestic pigs (Sus scrofa) (25 and 50 lb) obtained from the veterinary school of the Université de Montréal were used in this study. The animals were anesthetized with chloral hydrate (0.4 mg/g body weight) and perfused through the carotid artery with lactated Ringer's solution (Abbott Laboratories, Montréal, Québec, Canada), followed by a fixative consisting of 1% glutaraldehyde and 1% formaldehyde in 0.08 M sodium cacodylate buffer containing 0.05% calcium chloride (pH 7.3). The lower and upper jaws were dissected, immersed in the same fixative for an additional 12 h at 4°C, and then cut in a bucco-oral plane in smaller segments using a diamond-coated band saw. These jaw segments were decalcified at 4°C in 4.13% disodium ethylenediaminetetraacetic acid (EDTA) until decalcification was completed (Warshawsky & Moore 1967). After extensive washing in 0.1 M sodium cacodylate buffer containing 5% sucrose, pH 7.3, some samples were postfixed with potassium ferrocyanide-reduced osmium tetroxide (Neiss 1984) and processed for embedding in Taab 812 epoxy resin (Marivac, Halifax, NS, Canada). Unosmicated samples were embedded in LR White resin (Mecalab, Montréal, Québec, Canada).

Light microscopy

Semithin survey sections (1 μm thick) were cut with a diamond knife on a Reichert Ultracut E microtome (C. Reichert, Optische Werke AG, Wien, Austria), stained with toluidine blue, and observed by light microscopy. The two pigs had deciduous and permanent teeth covering a broad range of tooth developmental stages. Only teeth at the very beginning of root development (i.e. shortly after crown morphogenesis was completed) were selected for this study. This resulted in a total of 16 tissue blocks that were used for ultrastructural and immunocytochemical analyses.

Ultrastructural analyses

Epon blocks were trimmed to include the region of interest (i.e. from the intact HERS to the cemento-enamel junction). Thin (80–100 nm) sections were cut with a diamond knife, contrasted with uranyl acetate and lead citrate, and examined in a JEOL JEM 2000FX-II transmission electron microscope (JEOL Ltd, Tachikawa, Japan) operated at an accelerating voltage of 80 kV.

Immunocytochemistry

The high-resolution protein A–gold technique (reviewed in Bendayan 1995) was used for the immunocytochemical localization of EMPs along the forming roots. All incubations were performed at room temperature. Thin tissue sections were mounted on formvar- and carbon-coated nickel grids. Sections from LR White-embedded tissues were floated for 10 min on a drop of 0.01 M phosphate-buffered saline (PBS), pH 7.3, containing 1% ovalbumin (Sigma Chemicals, St Louis, MO, USA) to saturate nonspecific binding sites. The grid-mounted sections were then transferred and incubated for 1 h on a drop of anti-rat ameloblastin antibody diluted 1:20 with PBS (courtesy of Dr P.H. Krebsbach, National Institutes of Health, Bethesda, MD, USA) (Lee et al. 1996). Sections of osmicated tissues embedded in Epon were first floated on a drop of sodium metaperiodate for 15 min and washed with distilled water. The epoxy or LR White sections were then incubated for 3 h on a drop of egg yolk chicken anti-rat amelogenin antibody diluted 1:150 with PBS (Chen et al. 1995), or sheep anti-porcine Bio-Gel peaks F and C affinity-purified amelogenin antibodies diluted 1:10 and 1:100, respectively, with PBS (courtesy of Dr H. Limeback, Faculty of Dentistry, University of Toronto, Ont., Canada) (Limeback & Simic 1990). Thereafter, the tissue sections were washed with PBS, floated again on PBS–1% ovalbumin for 10 min, and incubated for 1 h on a drop of the corresponding polyclonal rabbit anti-chicken IgG (Cappel, Scarborough, Ont., Canada) or anti-sheep IgG (Cappel) secondary antibody.

After incubations with primary or secondary antibody, the grids were rinsed with PBS, floated on PBS–1% ovalbumin for 10 min, and incubated with protein A–gold complexes prepared using gold particles of approximately 12 nm diameter (Frens 1973). After rinsing with PBS and distilled water, the sections were grid-stained with uranyl acetate and lead citrate and examined in the transmission electron

microscope. As controls, sections were incubated with protein A–gold alone, nonimmune serum, secondary antibodies, or unrelated antibodies.

Results

Light microscopy

At very early stages of root formation, the apical portion of HERS, the so-called epithelial diaphragm, was very elongated. The epithelial cells stood out against the mesenchymal cells of the dental follicle proper and the papilla by their basophilia (Fig. 1a). Before HERS began to disintegrate, the inner epithelial cells were flat (Fig. 1a). Disintegration commenced shortly after the first layer of mantle dentin matrix was deposited (Fig. 1b). At the same time, the epithelial cells began to change their shape from elongated (Fig. 1a) to ovoid or polygonal (Fig. 1b–d) and occupied an increasingly widening territory adjacent to the root surface. Their small intercellular spaces became filled with a light-staining extracellular matrix. A thin seam of similar looking matrix was present between the epithelial cells and the now mineralized root dentin (Fig. 1c, d). Cell invasion from the dental follicle proper towards the root surface was morphologically not discernible.

In a few tissue samples, the inner cells of HERS at its apical-most portion were polarized and elongated (Fig. 1e). They thus assumed the morphology reminiscent of young ameloblasts. Cells resembling those of the stratum intermedium and stellate reticulum (Fig. 1e) occupied the space between the inner and outer layers of HERS. Further coronally, disintegration of HERS appeared to be delayed and regions with no apparent disintegration (Fig. 1f) alternated with regions displaying delayed disintegration (Fig. 1g, h). In one tissue sample, a thin and dark-staining matrix layer with an irregular contour was observed between such an island of elongated epithelial cells and the dentin surface (Fig. 1f). It must be clearly pointed out that this matrix layer was isolated, i.e. apical and coronal to it, HERS disintegrated and cementogenesis initiated. Incubations with antibodies against amelogenins resulted in a strong labeling of this particular matrix layer (not shown). While an early stage of delayed HERS disintegration is illustrated in Fig. 1g, a more advanced developmental stage is shown in Fig. 1h. At both stages, the space occupied by the disintegrating HERS cells was unusually wide. In analogy to what has been described for Fig. 1c, d, a light staining extracellular matrix had formed both between the epithelial cells and along the dentin surface.

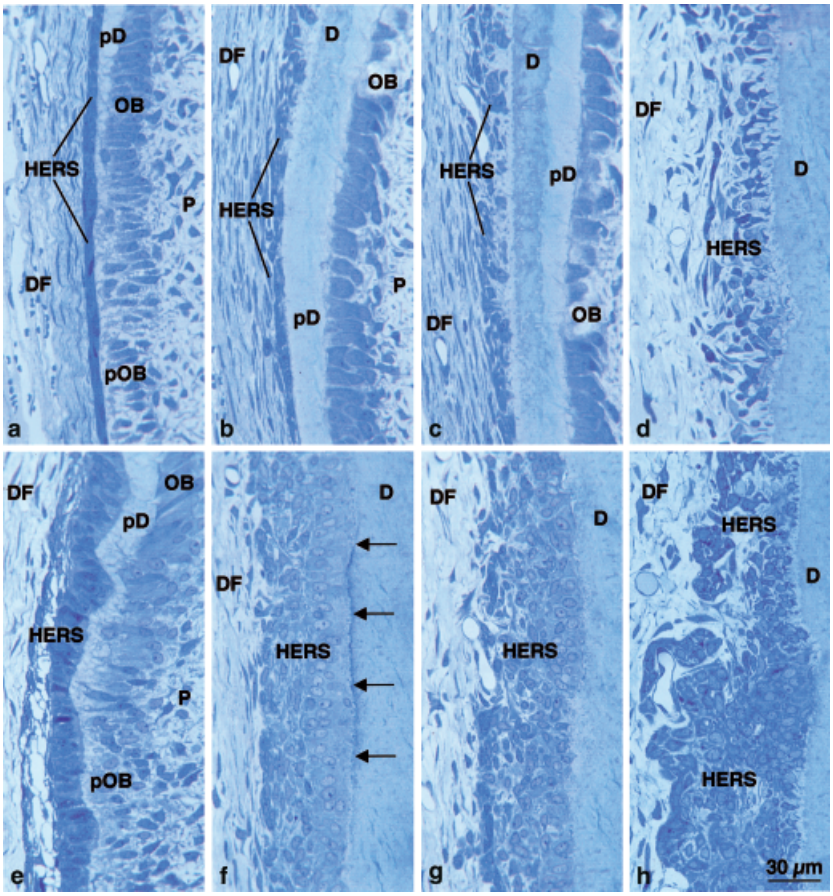

Fig. 1. Light micrographs illustrating the development of Hertwig's epithelial root sheath (HERS) before (a), at the beginning of (b), and following its disintegration (c, d). Note the change of cell shape from flat in the intact HERS (a) to round or polygonal (b–d). The dissipating HERS cells occupy a widening space that becomes filled with an extracellular matrix. In a few samples, the inner cells of HERS are elongated and oriented at a right angle to the root surface (e). (f) shows an isolated region where HERS did not disintegrate. Its inner cells assume an ameloblast-like morphology and a thin enamel matrix layer (as verified by immunocytochemistry) is present (arrows). Apical and coronal to such regions, HERS' disintegration has occurred and the dissipating cells occupy a wide compartment between the root surface and the dental follicle proper (DP) (g, h). OB, odontoblasts; P, pulp; pD, predentin; pOB, preodontoblasts.

Transmission electron microscopy

In most samples, the epithelial cells in the intact HERS were elongated with their long-axis oriented parallel to the root surface (Fig. 2a). Desmosomal cell junctions, although sparsely distributed, were regularly observed and tonofilaments were not abundant. Epithelial cell nuclei were slender to ovoid. The cytoplasmic to nuclear area ratio was low and the rough endoplasmic reticulum was poorly developed (Fig. 2a). As the epithelial cells developed a higher cytoplasmic to nuclear area ratio, HERS increased in thickness. A prominent Golgi apparatus and well-developed cisternae of rough endoplasmic reticulum were also apparent at this stage. The inner and outer basement membranes remained intact (Fig. 2b). Thereafter, slender cell processes began to penetrate the inner basement membrane, while the outer one remained intact (Fig. 2c). Finally, broad cytoplasmic extensions projected from the epithelial cell bodies towards the root surface breaking down the continuity of the inner basement membrane (Fig. 2d). Their nuclei, which initially had a higher

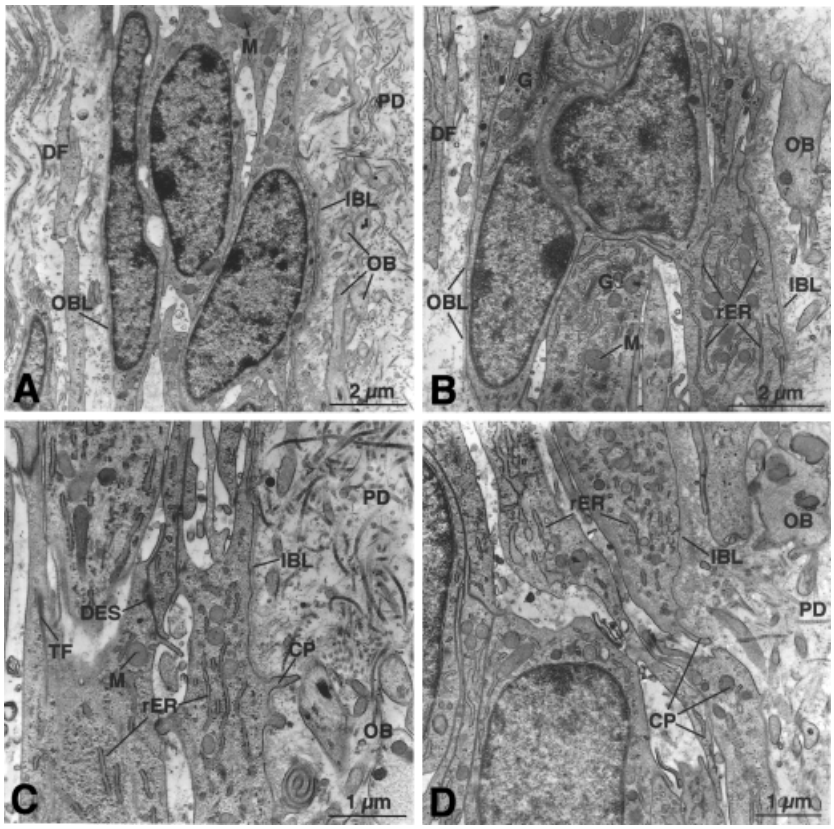

Fig. 2. Transmission electron micrographs illustrating the development of Hertwig's epithelial root sheath (HERS) cells from the intact to the disintegrated stage. (a) In the intact HERS, the epithelial cells are slender with poorly developed cytoplasmic organelles. The inner (IBL) and outer basal laminae (OBL) are intact. (b) In the next stage, the epithelial cells have developed a higher cytoplasmic to nuclear area ratio (compare with (a)). A prominent Golgi apparatus (G) and abundant rough endoplasmic reticulum (rER) are now apparent. The IBL and OBL remain intact. (c) Later in time, tiny epithelial cell processes (CPS) penetrate the IBL. (d) Broad CPs break down the continuity of the IBL. DF, dental follicle proper; DES. desmosome; M, mitochondrium; OB, odontoblasts; PD, predentin; TF, tonofilaments.

heterochromatin content (Fig. 2a), became euchromatic and less elongated (Fig. 2d). Following this, the epithelial cells began to dissipate, occupying an increasingly widening compartment between the root surface and the dental follicle proper. The intercellular spaces became filled with banded collagen fibrils (Fig. 3a). Single or neighboring cells formed bay-shaped surface indentations filled with crosscut collagen fibrils (Fig. 3a). The Golgi complex and cisternae of the rough endoplasmic reticulum continued to be prominently expressed and the morphology of the nuclei was multiform (Fig. 3a). While the cells now assumed morphologic features reminiscent of cementoblasts, desmosomes and tonofilaments remained discernible in most cells (Fig. 3a, b). In a few cells, these typical epithelial features were not detectable.

Immunocytochemistry

While immunolabeling for ameloblastin was not observed along the forming roots in any of the samples, amelogenin protein expression was occasionally detected. All anti-amelogenin antibodies yielded a similar labeling pattern. Labeling for amelogenin was restricted to tissue samples displaying the earliest stages of root formation (i.e. close to the cemento-enamel junction where the break-up of HERS occurred for the first time). Gold particles were associated with round, organic matrix deposits of variable sizes that were sporadically observed and randomly distributed along the forming root surfaces (Fig. 4a, b) and at a distance from it among the dissipated epithelial cells (Fig. 4c). Thus, regions with a dense accumulation of patches alternated with regions lacking any labeled extracellular matrix elements. The amelogenin-reactive material was co-localized with the collagenous matrix of precementum (Fig. 4a–c).

While in the majority of cells, cisternae of the rough endoplasmic reticulum were abundant and the Golgi apparatus was prominent, labeling for amelogenin in the Golgi apparatus and secretory granules was observed in a few cells only (not shown). While this intracellular labeling was observed for all anti-amelogenin antibodies, the anti-ameloblastin antibody did not result in any intracellular labeling. Control incubations of the matrix of the immature crown enamel, however, did result in intense gold particle labeling for all antibodies used and the labeling patterns were compatible with those observed in a recent study (Nanci et al. 1998). Incubations of tissue sections from more advanced stages of root development, which were used for comparison, did not result in any detectable intra- or extracellular immunolabeling in the region where cementogenesis initiated.

Discussion

In all cases, sections incubated under negative control conditions only showed very few, randomly distributed gold particles. The present study demonstrates that the very early stages of root formation in the pig show a combination of special features that were not described in its present form in other species thus far. These particularities include the (1) morphology of the inner cell layer of HERS; (2) disintegration process of HERS; (3) association of epithelial cells with the developing precementum matrix; and (4) presence and spatial association of EMPs with the developing precementum matrix. It must be noted that these observations were restricted to the very early stages of root formation. More advanced root formation stages, which were used for comparative purposes, did reveal the same features with the following exceptions. The inner cells of HERS did not assume the morphology of preameloblasts in any of these samples, and EMPs were not immunodetected. Furthermore, these tissue samples showed that a mixed type of cementum consisting of intrinsic and extrinsic fibers with embedded cementocytes covered the entire root surface. This is in line with observations made by Furseth (1970), but contrasts with findings in mice, rats, and humans

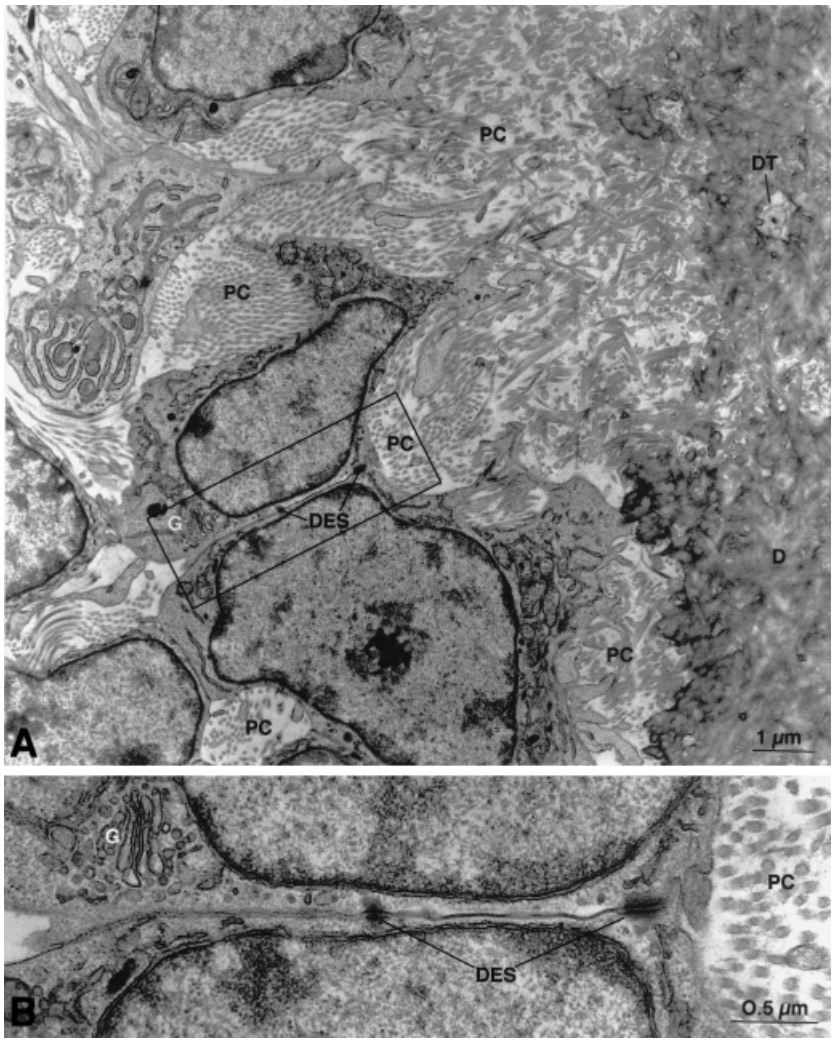

Fig. 3. Transmission electron micrographs showing the root surface after completion of HERS' disintegration and at the beginning of precementum (PC) formation. (a) The dissipated epithelial cells assume the typical morphology of cementoblasts, i.e. they have a prominent Golgi apparatus (G) and abundant rough endoplasmic reticulum and have formed bay-shaped surface indentations filled with crosscut precementum collagen fibrils (PC). Almost the entire extracellular space around the cells is filled with collagen fibrils (PC). Desmosomes (DES) are seen connecting neighboring cells. (b) shows a higher magnification of the desmosomal cell contacts (DES) seen between the two central cells in (a). D, dentin; DT, dentinal tubule.

where acellular extrinsic fiber cementum prevails on the cervical half of the roots (for reviews, see Bosshardt & Schroeder 1996, Bosshardt & Selvig 1997).

Morphology of the inner layer of HERS

The internal cells of HERS in rats, mice, dogs, and humans are usually cuboidal in shape or oriented parallel to the root surface (Owens 1978, Heritier & Fernandez 1981, Cho & Garant 1988, Bosshardt et al. 1998). While this morphology was also observed in the pig, other tooth samples revealed short and columnar cells in the internal layer of HERS. This morphology is reminiscent of the internal cells of the enamel organ, which differentiate into ameloblasts. It is therefore concluded that the inner cells of HERS occasionally assume the phenotype of preameloblasts during the very beginning of root formation in the pig. The observation of a narrow intermediate zone consisting of stellate reticulum- and intermediate-like cells in the HERS at early root developmental stages supports the contention that HERS does occasionally retain structural features of the enamel organ. These findings suggest that in these instances, the disintegration process of HERS was lacking or delayed. That HERS cells are able to enter the pathway of ameloblast differentiation is known from the formation of ectopic enamel deposits (Bosshardt & Nanci 2003).

The disintegration process of HERS

A decisive developmental step that distinguishes root from crown formation is that the inner enamel epithelial cells normally do not differentiate into ameloblasts. Instead, HERS disintegrates and its cells withdraw from the root surface to form the epithelial rests of Malassez, which persist as an epithelial network in the periodontal ligament. The initiation of cementogenesis is at least temporally linked to the disintegration process (for reviews see Bosshardt & Schroeder 1996, Bosshardt & Selvig 1997). However, precise knowledge about how disintegration is initiated, how epithelial cells withdraw from the root surface, and how migrated epithelial cells reconstitute to form the network of the epithelial cells of Malassez is missing.

The observations made in the pig provide new insights into the process of epithelial disintegration and further cell development. There were no morphological signs observed that would allow us to conclude that a complete dislocation of all HERS' cells from the root surface into the periodontal ligament space occurred. This was confirmed by transmission electron microscopy showing cells with desmosomes, tonofilaments, and/or remnants of the basement membrane remaining on the root surface. There were also no morphological details discernible that are indicative of a migration of dental follicle cells towards the root surface. Migration of dental follicle cells towards the root and subsequent differentiation into cementoblasts is, however, part of traditional thinking (for reviews, see Bosshardt & Schroeder 1996, Cho & Garant 2000). Based on our observations in the pig, we suggest that cells of the disintegrating HERS disseminate and thereby occupy an increasingly widening territory next to the root surface. The leaving behind of epithelial cells on the root has also been observed in rats (Lester 1969, Bosshardt & Nanci 1998, 2000, Bosshardt et al. 1998).

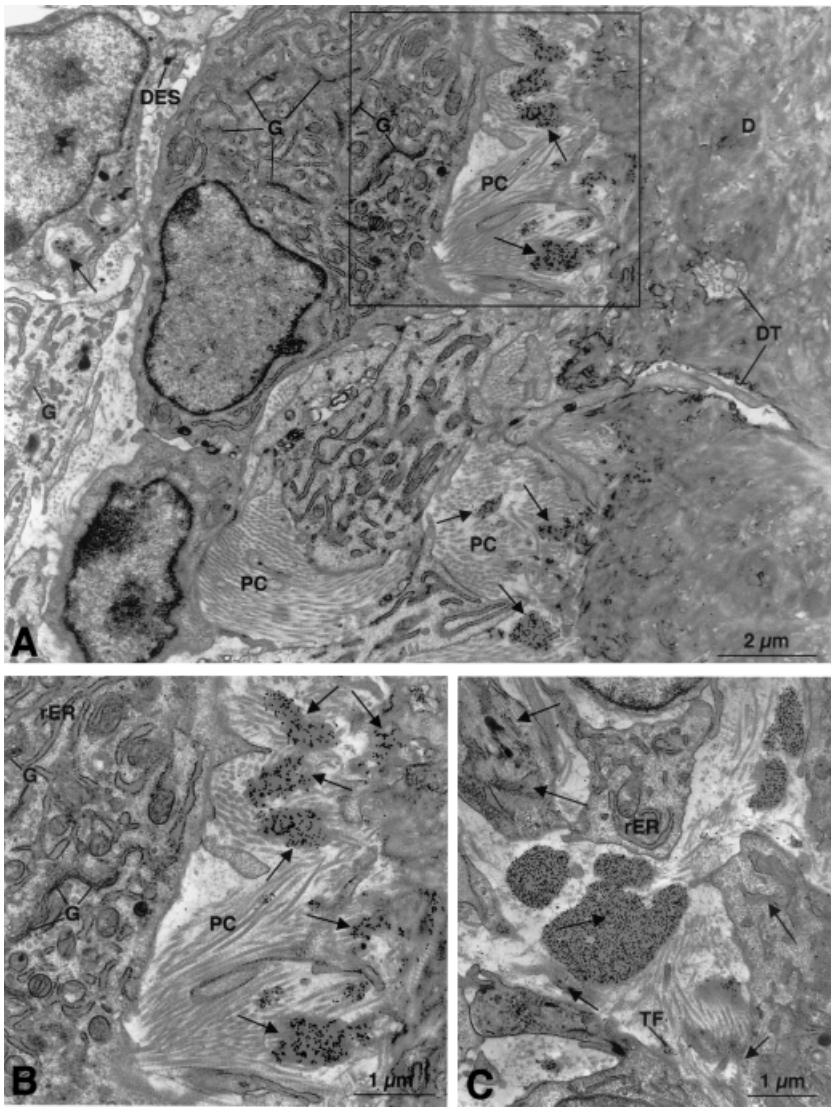

Fig. 4. Immunocytochemical preparation with an anti-amelogenin antibody at the very beginning of precementum (PC) formation. The area outlined in (a) corresponds to (b). (a, b) Round organic matrix deposits labeled with gold particles (arrows) are sporadically observed and randomly distributed along the dentin (D) surface where they co-localize with the collagenous precementum matrix (PC). The associated cells have a well-developed Golgi apparatus (G) and abundant rough endoplasmic reticulum (rER). (c) Labeled matrix deposits (arrows) are also found at a distance from the root surface where they co-localize with collagen fibrils distributed among epithelial cells. DES, desmosomes; DT, dentinal tubules; TF, tonofilaments.

Association of epithelial cells with the developing cementum matrix

The presence of collagen fibrils among the disintegrated cells of HERS signalizes the initiation of cementogenesis. Cementum matrix is produced by cementoblasts, which are widely believed to descend from the dental follicle proper (Ten Cate 1998). Since the observations made in the pig are not compatible with the traditional view of cementogenesis, they need to be discussed in greater detail.

A striking observation in HERS cells was the development of the full cytoplasmic armamentarium required for protein synthesis and export. Lester (1969), Owens (1978, 1980), and Slavkin et al. (1989b) already noticed in HERS' cells organelles suggestive of secretory activity. Slavkin et al.'s (1989b) interpretation of the presence of secretory granules in HERS' cells was that they produce EMPs. The observations made in the pig suggest that this is not the only possibility. HERS' cells may have produced other proteins such as cementum matrix constituents (Lester 1969, Owens 1978, Bosshardt & Nanci 1998, 2000, Bosshardt et al. 1998). In fact, the large number of epithelial cells showing a well-developed rough endoplasmic reticulum and Golgi apparatus but lacking intracellular labeling for EMPs supports this proposition. The continued expression of these cytoplasmic organelles and the absence of intra- and extracellular labeling for EMPs at more advanced stages of root development strengthen this proposal. The data in the pig clearly show that the epithelial cells assumed the morphology of cementoblasts (see Fig. 3a, 4a) and that their widening intercellular spaces become filled with banded collagen fibrils. The formation of bay-like surface indentations filled with crosscut collagen fibrils, as observed in the present study in epithelial cells, is a typical feature of fiber-forming cells like cementoblasts (Bosshardt & Schroeder 1991, 1992) and other connective tissue cells (Birk & Trelstad 1984, 1986). However, the expression of rudimentary tonofilaments (i.e. cytokeratins) and desmosomes in the cells resembling cementoblasts indicates an epithelial ancestry. Why an epithelial cell assumes the morphology of a connective tissue cell can be explained by a phenotypic conversion. This phenotypic conversion, known as epithelial–mesenchymal transformation, plays an important role in embryogenesis and neoplastic development (Hay 1991, 1995, Thiery & Chopin 1999). The presence of mesenchymal cells among disintegrated HERS' cells is usually interpreted as a sign for cell migration from the dental follicle proper towards the root surface (Cho & Garant 1988). Alternatively, cells lacking any vestigial epithelial characteristics among cells still displaying epithelial features may represent cells that have completed the process of epithelial–mesenchymal transformation. Thus, the findings in the pig lend support to the concept that cementoblasts originate from the HERS (Thomas & Kollar 1988, Bosshardt 1993, MacNeil & Thomas 1993, Bosshardt & Schroeder 1996, Webb et al. 1996, Bosshardt & Nanci 1997, 1998, 2000, Bosshardt et al. 1998, Lezot et al. 2000, Oishi et al. 2000).

Detection of EMPs along the forming root

EMPs along the root were often associated with the so-called intermediate layer of cementum (Lindskog 1982a, b,

Lindskog & Hammarström 1982, Slavkin et al. 1989b, Sasano et al. 1992, reviewed in Bosshardt & Schroeder 1996). A perusal of the literature, however, indicates that there is no evidence for the existence of an EMP-containing matrix layer on the root dentin. Schonfeld & Slavkin (1977), for instance, used an inappropriate animal model (see Grevstad & Selvig 1982). In another oftencited paper, Lindskog & Hammarström (1982) used the amino acid tryptophan to distinguish between enamel and collagenous matrices. However, tryptophan, although found in amelin (Cerny et al. 1996), is not a specific marker for EMPs. Compared with amelin, it is to equal or even higher relative amounts are also found in α1(I), α2(I), α1(IV), and α2(IV) collagen and in osteopontin (Oldberg et al. 1986, Butler 1989). Recent studies using in situ hybridization and immunolocalization techniques were also not able to support the intermediate cementum theory (Fong et al. 1996, Thomas et al. 1997, Bosshardt & Nanci 1998, 2000, Bosshardt et al. 1998, Fong & Hammarström 2000).

Using a high-resolution technique for protein detection, the present investigation did not detect any immunolabeling for ameloblastin along the forming roots. Control incubations of the crown enamel matrix did, however, result in a labeling pattern typical of ameloblastin (Nanci et al. 1998). Thus, the lack of ameloblastin-labeling may not be attributed to the antibody. During crown development, ameloblastin is immunodetected in the forming extracellular matrix after amelogenin and only when mineralization is about to start (Nanci et al. 1998). The secretion of ameloblastin into the extracellular milieu may not have started yet along the porcine root or it may be under the detectability threshold of the labeling method used. Alternatively or in addition, the parent ameloblastin molecule may be rapidly processed after its extracellular release (Nanci et al. 1998).

In contrast, all antibodies against amelogenin produced a positive reaction along the forming root surface in most teeth. Control sections containing crown tissues gave a positive reaction over the enamel matrix as well. Immunolabeling for amelogenin was always restricted to very early stages of root development. In other words, in none of the tissue samples, which had exceeded the very earliest stage of root formation and that were used as controls, could any gold particle labeling for amelogenin be detected in association with the forming root. The distribution pattern of amelogenin along the cervical-most portion of the root was very sporadic. The observed association of gold particles with discrete matrix patches corresponds to what has been reported for the presecretory stage of amelogenesis (Nanci et al. 1998). Unlike in amelogenesis, however, the size and distribution pattern of these enamel matrix patches were totally unpredictable along the cervical-most root portion in the pig. In addition, the labeled matrix patches were not only observed at the apical pole and lateral of the epithelial cells. Instead, they were also present far away from the root surface where they were randomly distributed among the dissipated cells. The deposition of a thin amelogenin-containing matrix layer along the root dentin was an exceptionally rare observation and can, therefore, not be used to support the intermediate cementum theory.

The present and other studies demonstrate that there is no consistent expression of EMPs along the forming root and among species (Slavkin et al. 1989b, Fong et al. 1996, Bosshardt & Nanci 1998, 2000, Fong & Hammarström 2000). Significantly, both gene and protein expression of EMPs were, however, not observed in association with acellular extrinsic fiber cementum (Luo et al. 1991, Bosshardt & Nanci 1998, 2000, Hu et al. 2001, present study). It is therefore difficult to perceive why a causal link between EMPs and the formation of acellular extrinsic fiber cementum (Hammarström 1997) should exist. Experimental data, such as analysis of root development in knockout mice, supporting this hypothesis are hitherto missing, and histologic examination of diseased human root surfaces treated with EMPs showed the formation of a repair tissue resembling cellular intrinsic fiber cementum (Sculean et al. 1999, 2000).

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