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Morphology of Malassez's epithelial rest-like cells in the cementum: transmission electron microscopy, immunohistochemical, and TdT-mediated dUTP-biotin nick end labeling studies

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Background and Objective: It is known that epithelial islands are embedded in the cementum during tooth root formation, but details of this process remain unknown. The purpose of this study was to investigate the dynamic characteristics of Malassez's epithelial rest cells in the cementum during tooth root formation in pigs *in vivo*.

Material and Methods: The first molars of 6-mo-old pigs were used in this study. Specimens were decalcified before being embedded in paraffin. Paraffin sections were investigated using TdT-mediated dUTP-biotin nick end labeling (TUNEL), immunohistochemical, and ultrastructural techniques.

Results: Malassez's epithelial rest cells were located close to the root surface at the apical one-third of the periodontal ligament, and epithelial clusters surrounded by distinct lamina cementia were sometimes observed in the cementum. TUNEL-positive cells were detected only in the cementum. Malassez's epithelial rest cells in the periodontal ligament were completely surrounded by basement membranes, but epithelial clusters in the cementum were only intermittently surrounded by such membranes. Cytokeratin-positive cells in the superstratum of the cementum were directly connected by cementocytes and by desmosome-like structures. However, organelles were scarce in the cytokeratin-positive cells in the substratum of the cementum, and the matrix of the cementum was deposited in the cells.

Conclusion: These results suggest that the majority of the fragmented Hertwig's root sheath remains in the periodontal ligament and that some cells, which are connected to cementoblasts, are embedded in the cementum and progress to apoptosis.

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It is known that Hertwig's epithelial root sheath functions in the induction and differentiation of dental papilla cells to odontoblasts. When the root dentine forms, the epithelial root sheath starts to disintegrate and secretes enamel-related proteins to the newly formed root dentin surface. Dental sac cells are then able to migrate on the dentin surface through the disintegrated regions of the epithelial root sheath, and they differentiate to cementoblasts following the induction of enamel-related proteins (1). Finally, the disintegrated root sheath generally remains as Malassez's epithelial rest (MER) cells in the periodontal ligament, although some are embedded in the cementum (2).

MER cells are generally known as resting cells because they express bcl-2 protein (an apoptosis inhibitor) throughout their lifetime (3). MER cells are characterized by condensed rounded nuclei with a high nuclear/cytoplasmic (N/C) ratio, Golgi complexes accompanied by vesicles, and a poorly developed rough endoplasmic reticulum (ER) in the cytoplasm. Furthermore, gap junctions and desmosomes are observed (4). It has been suggested that these cell islands have functions that might support the homeostasis of the periodontal ligament (5), induce cementum formation (6), induce and form the nerve (7), and/or inhibit the resorption of the tooth root (8). In contrast, when stimulated by inflammatory cytokines, MER cells can grow and differentiate into the epithelium of a periapical cyst (9) or a periodontal pocket (5). It is also known that some of the disintegrated root sheath remains embedded in the cementum of pigs (10) and rats (2). The embryological development of the oral cavity in pigs has been recognized to be very similar to that found in humans – in fact, it is more similar to humans than to any other animal. Hence, it seems that the use of pigs is an excellent approach to further develop dental research. However, the details of morphology are still unclear.

The purpose of this study was to investigate the morphological characteristics of the epithelial cell clusters in the cementum of pigs using TdT-mediated dUTP-biotin nick end labeling (TUNEL), immunohistochemical, and ultrastructural techniques.

Material and methods

Experimental animals

Three, 6-mo-old Landrace-Large White-Duroc pigs (120 kg body weight) were used in these experiments. Mandibulae were removed within 3–4 h of slaughter, and the first molars with the surrounding tissues were used as specimens. This study was performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Light microscopic techniques

The mandibular artery was exposed at the foramen mandibula, a polyethylene tube (1.09 mm diameter) was inserted into the artery, and fixation was performed by perfusion for 1 h using 4% (v/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Tissues were then sliced using a rotary saw cooled with running water, and were immersed in the same fixative solution, described above, for 48 h at 4°C. Specimens were dehydrated in a graded series of ethanol, and were decalcified in 10% (w/v) EDTA in 0.1 M phosphate buffer (pH 7.2) for 3 wk at 4°C. After decalcification, each specimen was embedded in paraffin, according to routine procedures, sections were cut to ≈ 5 μm thick, and were then stained by hematoxylin and eosin.

Immunohistochemical techniques

For immunohistochemistry, the streptavidin–biotin–peroxidase complex method was used with a Histofine kit (Nichirei, Tokyo, Japan). Antibodies to cytokeratin (CK; a cocktail of CK5 and CK8; Progen Biotechnik GmbH, Heidelberg, Germany), which reacts with squamous epithelium (at a dilution of 1 : 50), and to vimentin (Vim; DAKO, Tokyo, Japan), which reacts with mesenchymal cells (at a dilution of 1 : 20), were used as primary antibodies.

Paraffin-embedded sections were deparaffinized with xylene and washed in 10 mM phosphate-buffered saline (PBS, pH 7.2). Sections were treated with 0.1% (w/v) trypsin (in 10 mM Tris-HCl buffer, pH 7.6; Gibco BRL, Grand Island, NY, USA) for 30 min at 37°C to enhance the activity of the antibodies. Sections were then treated at room temperature with 0.3% (v/v) H_2O_2 in methanol to block endogenous peroxidase. After washing in PBS, samples were incubated overnight with primary antibodies at 4°C. Reactions were visualized using 0.05% (v/v) diaminobenzidine (DAB) and 0.005% (v/v) H_2O_2 in 0.05 mM Tris-HCl buffer (pH 7.2), and were counterstained with hematoxylin.

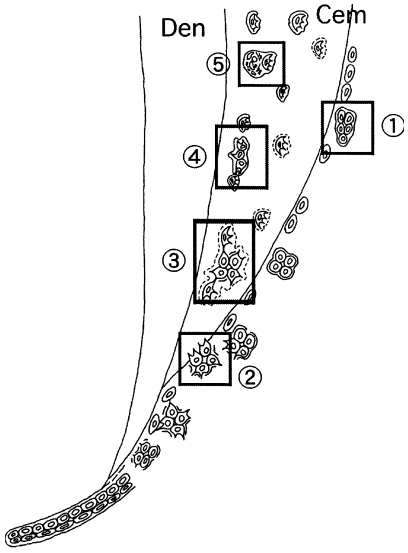
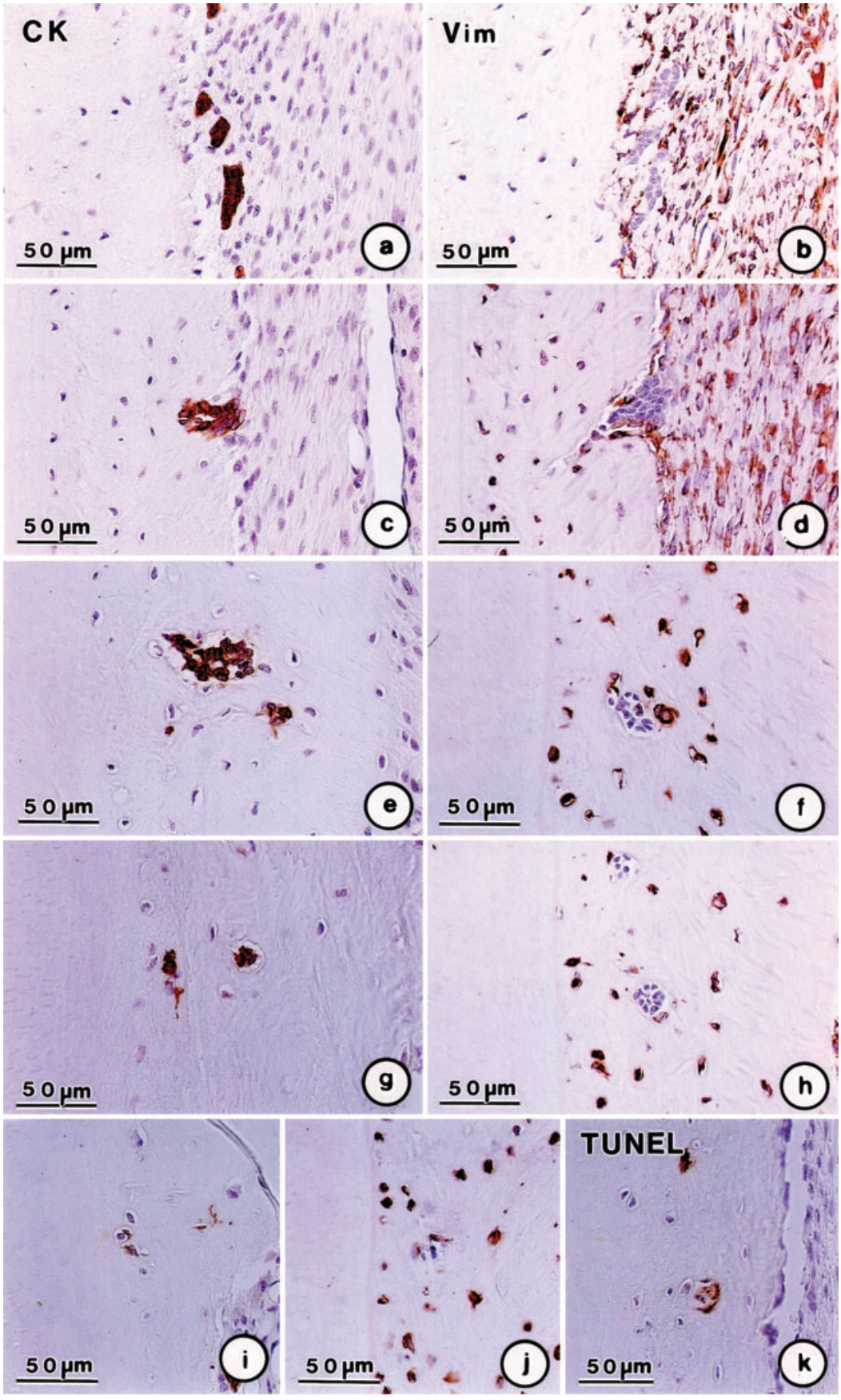


Fig. 1. Observation areas in the periodontal ligament and in the cementum. Malassez's epithelial rest (MER) cells remaining in the periodontal ligament (area 1) and embedded in the cementum (areas 2–5). Embedded clusters are located in the superstratum (areas 2 and 3) and the substratum (areas 4 and 5) of the cementum.

TUNEL method

The TUNEL method was used to detect apoptotic cells. Paraffin-embedded sections were deparaffinized with xylene, incubated for 20 min with 16.2 μg/ml proteinase K (in 10 mM Tris-HCl buffer, pH 7.4; Boehringer Mannheim, Mannheim Germany) at room temperature, and were then washed in distilled water. Endogenous peroxidase was inactivated in TdT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TUNEL
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buffer (30 mM Trizma base, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride; Gibco BRL) for 2 min at room temperature. Slides were then incubated in TdT (0.3 equivalent units/ml) and biotin–dUTP (0.04 nmol/μl; Gibco BRL) in TdT buffer at 37°C for 90 min. Reactions were terminated by transferring the slides to 300 mM sodium chloride, 30 mM sodium citrate (TB buffer) and washing them in PBS three times, for 10 min each, at room temperature. After rinsing in PBS, the slides were incubated with 10% (v/v) normal rabbit serum for 10 min to block nonspecific reactions. The slides were again rinsed in PBS, and were then incubated with peroxidase-conjugated streptavidin (Histofine SAB-PO kit; Nichirei) for 30 min at room temperature. After further washing in PBS, the slides were incubated in DAB and counterstained with hematoxylin.

Electron microscopic techniques

Mandibulae were perfused, as in the light microscopy technique described above, with 20 ml of modified Karnovsky's fixative [at a final concentration of 2% (v/v) paraformaldehyde and 2.5% (v/v) glutaraldehyde in 0.24 M Sorensen's phosphate buffer solution (pH 7.2)]. After decalcification with 10% (w/v) EDTA in 0.1 M phosphate buffer (pH 7.2) for 3 wk at 4°C, samples were washed with 0.12 M Sorensen's phosphate buffer and post-fixed in 2% (w/v) osmium tetroxide in the same buffer for 2 h. After the samples were block stained with 2% (w/v) uranyl acetate in 10% (v/v) ethanol for 20 min, they were dehydrated in a graded series of ethanol and embedded in Epon 812. Ultra-thin sections were cut at ≈60 nm thick, specimens were stained with uranyl acetate and lead citrate, and then observed using a HITACHI 7100 transmission electron microscope (Hitachi, Tokyo, Japan).

Results

Light microscopic observations

The tooth root formation of the mandibular 1st molars of 6-mo-old pigs was not complete. Hertwig's epithelial root sheath was seen as a funicular proliferation in the apical portion. After the formation of hard tissue, the majority of the disintegrated root sheath was left in the periodontal ligament (Fig. 1; area 1) as MER cells, and some MER cells were also embedded in the cementum (Fig. 1; Areas 2–5). Epithelial clusters in the cementum were seen in the superstratum (areas 2 and 3) and in the substratum (areas 4 and 5). These cells all immunostained strongly for CK in the cytoplasm. Each MER consisted of 10–20 cells, usually oval- or round-shaped, in the periodontal ligament located close to the cementum. MER cells were frequently seen at the cervical portion, but were sparse at the apical portion. Cells in the cementum were irregularly shaped compared with those seen in the apical portion (Fig. 2C,E,G,I). Cells in the cementum located near the periodontal ligament resembled the MER (Fig. 2C), but gradually decreased in number and degenerated close to the center of the root. The number of epithelial cells in the superstratum of the cementum increased compared with the number of epithelial cells in the cementum. Epithelial clusters in the cementum consisted of surrounding CK-positive cells (Fig. 2G,I). Those in the superstratum were clearly surrounded by the cementum wall (Fig. 2C,E), while those in the substratum were not (Fig. 2B).

Immunostaining for Vim was also positive for fibroblasts and cementoblasts in the periodontal ligament (Fig. 2B), and for cementocytes in the cementum (Fig. 2D,F,H,J). These cementocytes were surrounded by distinct lacuna cementea, with or without epithelial cells. Cementocytes were always observed around epithelial clusters that were negative for Vim.

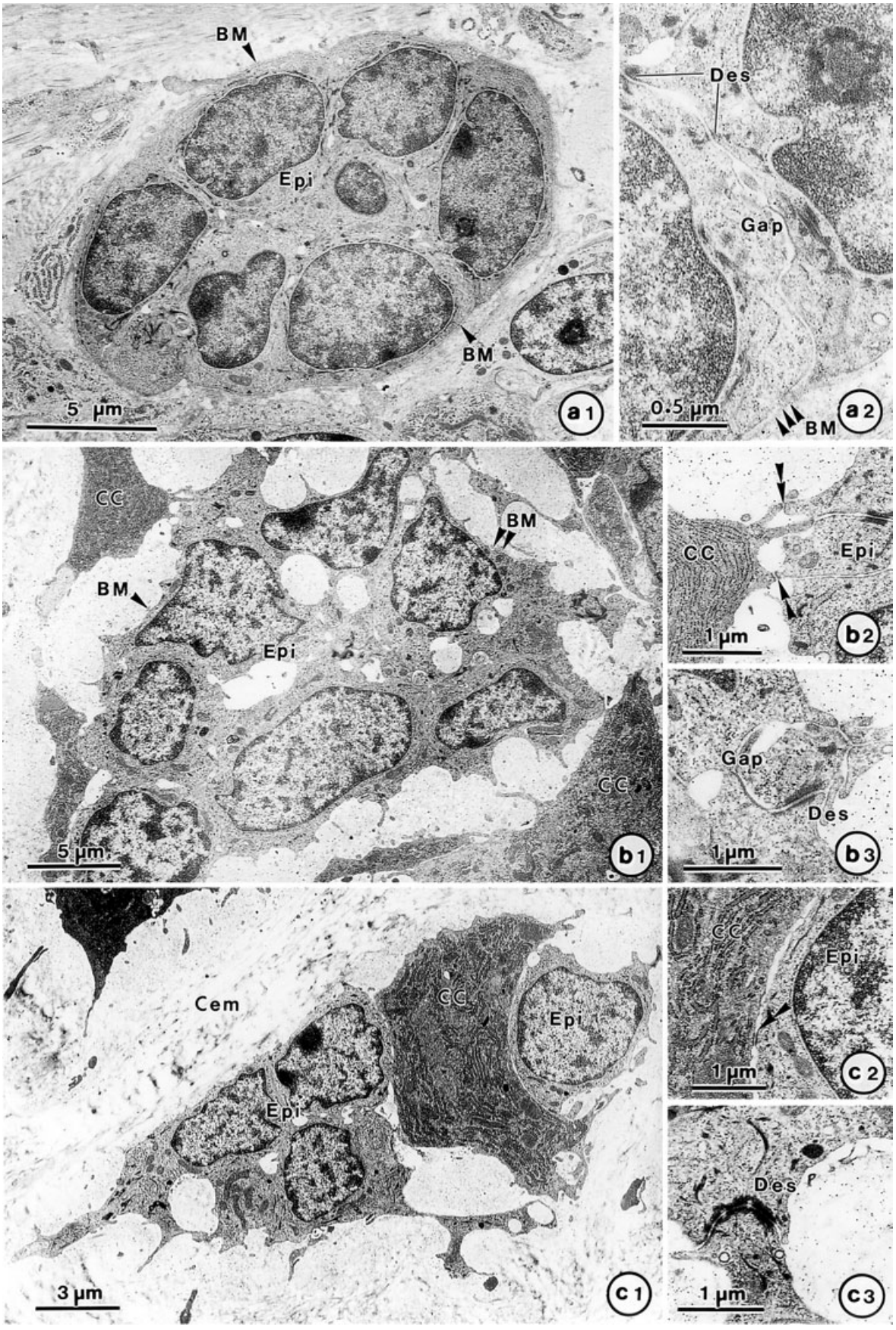
MER cells were negative for TUNEL staining. However, cells of epithelial clusters located deep in the cementum were positive for TUNEL staining and also immunostained for CK (Fig. 2K).

Electron microscopic observations

MER cells in the periodontal ligament were completely surrounded by a basement membrane, and the N/C ratio was quite high (Fig. 3A,1). The nuclei were oval- or round-shaped with condensed heterochromatin. Tonofilaments and relatively abundant mitochondria, poorly developed rough ER, and Golgi apparatus were observed in the cytoplasm. Desmosomes and gap junctions were frequently observed between cells (Fig. 3A,2).

Cell clusters in the cementum near the periodontal ligament were surrounded intermittently by a basement membrane. Several cells were in aster or oval shapes with many cell processes. The N/C ratio was high, and the

Fig. 2. (Overleaf) Immunohistochemical staining for cytokeratin (CK) and vimentin (Vim), and TdT-mediated dUTP-biotin nick end labeling (TUNEL). Area 1: Malassez's epithelial rest (MER) cells, close to the cementum surface in the periodontal ligament, as observed by CK staining. They consist of several types of epithelial cells, oval- or round-shaped (A). Fibroblasts in the periodontal ligament and cementocytes are positive for Vim, but MER cells are negative (B). Area 2: an epithelial cluster is embedded between the periodontal ligament and the cementum, and is positive for CK (C). Some cells around these clusters are negative for CK, but are positive for Vim (D). Area 3: an epithelial cluster is completely embedded in the cementum, and epithelial cells are positive for CK. A cell cluster is surrounded by lacuna cementea (E). Some cells around the epithelial cell cluster are positive for Vim; these cells exist in the lacuna cementea with epithelial cells (F). Area 4: epithelial cells positive for CK are embedded in the cementum matrix, and lacuna cementia are indistinct around the cluster (G). Cells around the epithelial cluster that are negative for CK are positive for Vim. These cells are close to the epithelial cells (H). Area 5: epithelial cells are positive for CK. The number of epithelial cells is decreased compared with other areas. Lacuna cementia are indistinct around epithelial cells and are embedded in the matrix of the cementum (I). Cells around the epithelial cells and cementocytes are positive for Vim (J). Cell clusters consisting of a few cells are detected by TUNEL (K).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oval- or aster-shaped nuclei had condensed heterochromatin, compared with those in the periodontal ligament (Fig. 3B,1). Relatively abundant mitochondria and poorly developed rough ER were observed in the cytoplasm. Intercellular spaces were wide, and few desmosomes and gap junctions were observed between cells (Fig. 3B,3). Cell clusters existed in the irregular cementum lumen and some existed with cementocytes, which were sometimes connected with desmosome-like structures (Fig. 3B,2).

Only a few basement membranes could be seen around the epithelial cells, which existed in the middle portion of the cementum (Fig. 3C,1). Several cells were deformed, and the heterochromatin was more condensed compared with cells near the periodontal ligament. Mitochondria and rough ER were observed in the cytoplasm. Desmosomes and gap junctions were observed between cells, and intercellular spaces were wide compared with those seen in the superstratum of the cementum (Fig. 3C,3). Cell clusters were surrounded by distinct lacuna cementea. Spaces between lumina of lacunae were wide, and collagen fibers were observed. Mineralized collagen and matrix on lumina lacunae were also observed. Epithelial cells were sometimes connected with cementocytes by desmosome-like structures, such as those seen near the periodontal ligament (Fig. 3C,2).

A basement membrane could not be seen around the epithelial clusters, which existed in the cementum near the dentin (Fig. 4A,1). The oval- or round-shaped nuclei had there was greater quantity of condensed heterochromatin compared with cells near the periodontal ligament. Some of the cells were embedded in the matrix of the cementum (Fig. 4A,3). Mitochondria and rough ER were observed in the cytoplasm; however, these organelles were scarce compared with those of cells in the superstratum of the cementum, and most had degenerated. Desmosomes and gap junctions were subsequently observed between cells (Fig. 4A,2). Cell clusters were surrounded by distinct lacuna cementea. These spaces between the lumina of lacunae were narrow, and parts of the cell membranes were indistinct because of the proximity of the lumina of lacunae.

Cell clusters in most of the substratum of the cementum had degenerated, and only denatured organelle-like structures were observed. Degenerated clusters were surrounded by lumina of lacunae (Fig. 4B,1 and 2).

Discussion

In this study, the root formation was not complete in mandibular 1st molars taken from 6-mo-old pigs. At that age, the functioning dentition consists of deciduous teeth and of permanent teeth (M1). The jaw consists of incisors, premolars, and 2nd molars. These permanent teeth had not erupted in all animals, and therefore we investigated their tooth development. It has generally been found that the systemic physiological and anatomical characteristics of pigs are highly similar to those of humans (11). Malassez characterized MER cell islands in the periodontal ligament, and showed that these were epithelial (12). Regarding the embryology of MER, it has been shown that the disintegrated Hertwig's root sheath remains in the periodontal ligament after cementum formation. In fact, it has been reported that it continues to exist after tooth eruption, although it can change in size with age (13). In this study, we observed epithelial clusters in the periodontal ligament, which agreed with previous studies.

It was reported by Lester (2) and by Furseth (10) that in rats and pigs, some MER cells are embedded in the cementum. Lester used rats to show that embedded epithelial cells exist in the intermediate cementum between the dentin and the cementum. Rats abound with cementum formation, in contrast to root dentin formation, and therefore epithelial islands are embedded between the dentin and the cementum (2). In contrast, the cementum at the cervical portion in pigs has an embedded cellular component, such as cementocytes (14). In our study, using pigs, we observed that epithelial cells in the cementum exist in various portions of the apical cementum, in agreement with the finding of Furseth (10). Epithelial cells located deep in the cementum were TUNEL positive, which suggests that these epithelial cells become apoptotic after they are embedded in the cementum and that some of them connect with cementocytes and protein located in epithelial cells residing in the cementum. This suggests, in turn, that cementum may be distinguished from bone tissue in terms of protein content. Furthermore, MER cells in the cementum are connected with cementocytes, suggesting that MER clusters are surrounded by cementum matrix that is secreted by

Fig. 3. (Overleaf) Transmission electron micrographs of areas 1, 2, and 3. Area 1: Malassez's epithelial rest (MER) cells in the periodontal ligament are completely surrounded by basement membranes, and these are separated from the mesenchymal tissue of the periodontal ligament; they consist of several types of epithelial cells (Epi), which are oval- or round-shaped. (A1). (A2) Magnification of a part of Fig. A1. Each epithelial cell is tightly connected by desmosomes and gap junctions (Gap). However, MER and the periodontal ligament are separated from the basement membrane (BM), and connecting structures are not seen. Area 2: cell clusters are partly surrounded by a basement membrane (BM). These cells are stellate and contain tonofilaments, rough endoplasmic reticulum, and mitochondria in the cytoplasm (B1); they are connected to mesenchymal cells by desmosome-like structures (B2: arrowhead). Wide intercellular spaces, desmosomes, and gap junctions are frequently seen between them (B3). Area 3: an interrupted basement membrane is seen around the epithelial clusters. Epithelial clusters are discontinuously surrounded by lacuna cementia (C1). Epithelial cells are directly connected to cementocytes by desmosome-like structures (double arrowheads). Cementocytes are observed with well-developed organelles in the cytoplasm (C2). Desmosomes and gap junctions are observed between epithelial cells (C3). BM, basement membrane; CC, cementocyte; Cem, cementum; Des, desmosomes; Epi, epithelial cells; Gap, gap junctions.

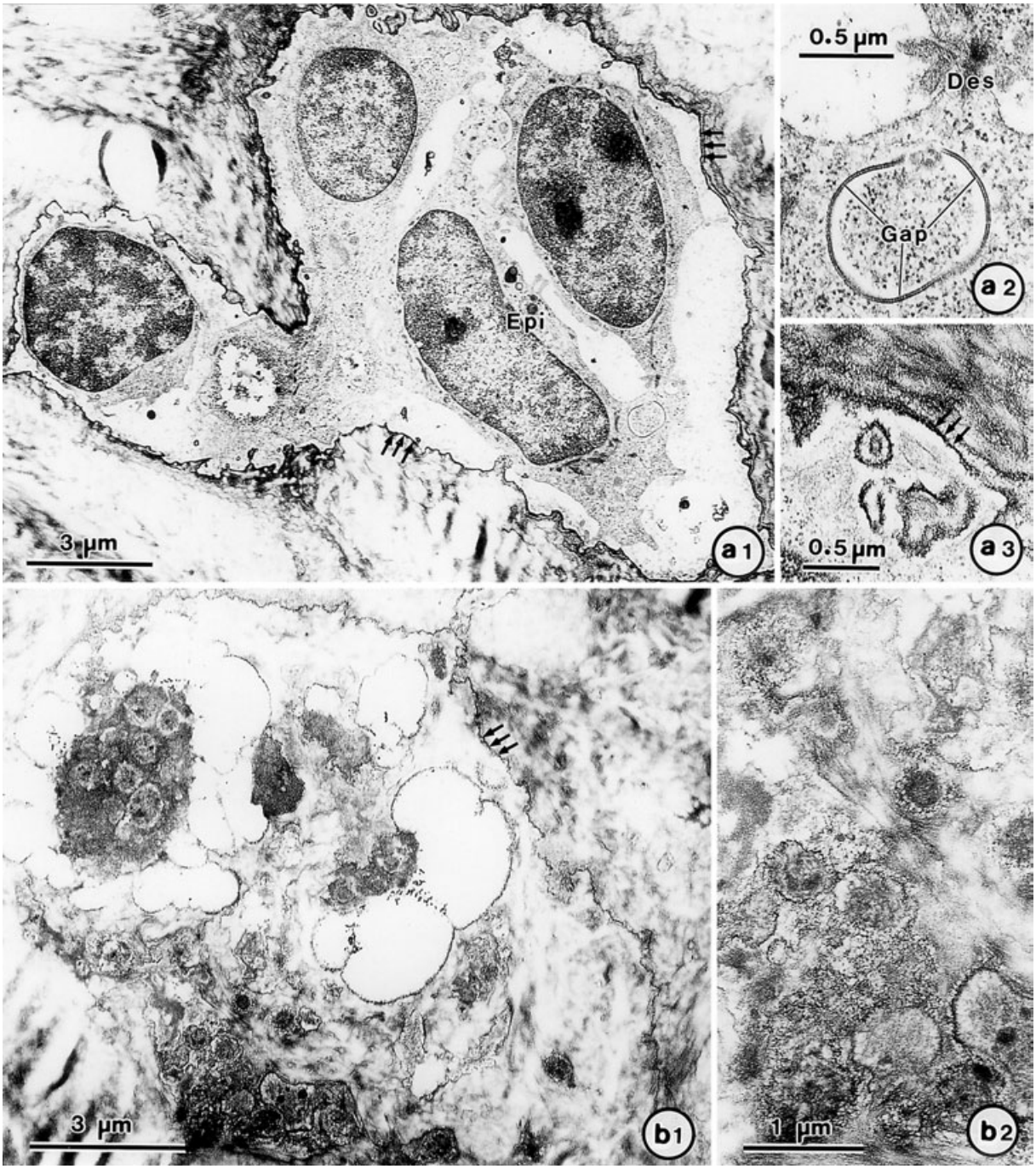

Fig. 4. Transmission electron micrographs of areas 4 and 5. Area 4: basement membranes are not seen around the epithelial cells. Organelles in the cytoplasm are scarce, but tonofilaments are well-developed (A1). However, desmosomes and gap junctions are still observed between epithelial cells (A2). These clusters are surrounded by distinct lacuna cementia (arrows), but are partly embedded in the matrix of the cementum (A3). Area 5: degenerated cells are observed surrounded by lacuna cementia (arrows) (B1). Organelles are not seen; however, swollen organelles and nuclear-like structures are observed (B2). Des, desmosomes; Epi, epithelial cells; Gap, gap junctions.

connecting cementocytes when the cementum is formed. Therefore, epithelial cells may be embedded in various portions of the cementum.

Lester reported that epithelial cells in the cementum of the root showed regressive changes of their nuclei (2). In this study, condensed nuclei and degenerated organelles were observed in the substratum cementum, in agreement with the finding of Lester (2). However, in the superstratum of the cementum, epithelial cells have cytoplasms containing mitochondria and rough ER, suggesting that these cells have cellular activities. In contrast, organelles are scarce and had degenerated at the substratum of the

cementum; thus, those epithelial cells may be dying by necrosis or apoptosis. They are embedded in the hard tissue, which suggests that alimentation results from cellular processes. MER cells in the substratum are oval-shaped and cellular processes are fewer, compared with MER cells in the superstratum, which suggests that these cells may have regressed and may be dying as a result of decreased alimentation, because of the disintegration of their cellular processes.

We observed that epithelial cells are embedded in the cementum only at the apical portion. The porcine cementum is constituted by the cellular cementum from the cervical portion to the apical portion. The apical portion cementum abounds with cementum formation, in contrast with the cervical portion. Hamamoto *et al*. reported that MER cells were left to migrate on the periodontal ligament from the dentin surface after the rupture of Hertwig's epithelial root sheath (15). Lester thought that this might result from their migration on the periodontal ligament, leaving epithelial islands embedded in the intermediate cementum (2). In our study, the amount of cementum formation is usually abundant at the apical portion. Therefore, cementoblasts around epithelial clusters may be caused by hyper-secretion of the cementum matrix during cementum formation. Hence, we suggest that the epithelial clusters are embedded in the cementum matrix before migration.

In this study, epithelial cells in the superstratum portion and in the middle portion of the cementum have cellular processes and are connected with cementocytes. It has been suggested that during cementum formation, epithelial cells secrete enamel-related proteins on the dentin surface and differentiate to cementoblasts from dental sac cells (1). Hamamoto *et al*. reported that epithelial cells are directly connected with cementoblasts during cementogenesis, and are involved in the cementum formation (15). Our study shows that epithelial cells are connected with cementocytes, and therefore we suggest that epithelial cells in the cementum interact with cementocytes and may be related to the maturation of the cementum.

Thus, in conclusion, we suggest that epithelial cells may die of apoptosis in the substratum area of the cementum where the amount of the cementum matrix increases. However, epithelial cells in the superstratum area of the cementum may be biologically active and may interact with the cementocytes.

Acknowledgements

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