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In vitro differentiation of epithelial cells cultured from human periodontal ligament

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Background and Objective: Alkaline phosphatase and noncollagenous bone proteins are produced prior to cementum formation. While it has been suggested that epithelial rests of Malassez are involved in cementum formation, little is known about the relationship between epithelial rests of Malassez and cementum formation. The purpose of the present study was to determine whether the epithelial rests of Malassez cells cultured from human periodontal ligament can produce alkaline phosphatase and noncollagenous bone proteins, such as osteopontin, osteocalcin and bone sialoprotein.

Material and Methods: An outgrowth of putative epithelial rests of Malassez cells was produced from periodontal ligament explant, and second passage cultures were used in the experiments. Human gingival epithelial cells and periodontal ligament fibroblasts were used as controls. The expression levels of amelogenin were analyzed by immunostaining and *in situ* hybridization. Furthermore, the expression levels of alkaline phosphatase and noncollagenous bone proteins were assessed by immunostaining and reverse transcription–polymerase chain reaction.

Results: Amelogenin, alkaline phosphatase and osteopontin proteins and their corresponding mRNAs were detected at high levels in putative epithelial rests of Malassez cells. Osteocalcin and bone sialoprotein were not expressed in putative epithelial rests of Malassez cells. Alkaline phosphatase and noncollagenous bone proteins were seen in periodontal ligament fibroblasts, but not in gingival epithelial cells.

Conclusion: Our results suggest that putative epithelial rests of Malassez cells cultured alone do not transform into maturing cells to form the cementum, but may play a potential role in the mineralization process.

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The epithelial rests of Malassez cells are odontogenic epithelial cells derived from Hertwig's epithelial root sheath cells, and amelogenin is the major enamel protein produced by ameloblasts at the differentiation and secretory stages (1). Localization of amelogenin has been observed in the enamel matrix, in normal ameloblasts, in odontogenic tumor cells, in the extracellular matrix *in vivo*, in mantle dentin and odontoblasts, and in Hertwig's epithelial root sheath cells (1–8). It has also been proposed that amelogenin acts as a signaling molecule in the formation of acellular cementum (9–12). Cytokeratin 14 is a known marker for ameloblasts in a developing tooth prior to the synthesis of amelogenin (13), and it is believed that interactions between cytokeratin 14 and amelogenin may play an important role in amelogenesis, enamel development and disease (14).

Alkaline phosphatase, and the major noncollagenous bone proteins osteopontin, osteocalcin (osteocalcin) and bone sialoprotein, are produced by osteoblastic cells and deposited into the bone matrix. The activity of alkaline phosphatase is required to generate the inorganic phosphate needed for hydroxyapatite crystallization.

Extracellular inorganic pyrophosphate is a potent inhibitor of hydroxyapatite formation. Alkaline phosphatase hydrolyzes extracellular inorganic pyrophosphate (15,16). Bone sialoprotein appears to be unique to mineralized connective tissue and also has the potential to mediate the initial formation of hydroxyapatite crystals (17–19). In contrast, osteopontin is highly phosphorylated and sulfated, but is unlikely to be a primary nucleator of hydroxyapatite formation because it appears before bone sialoprotein as a marker of an early stage of bone formation during the regenerative processes (20). Moreover, osteopontin activity has been shown to inhibit mineralization (3,20–23), and osteopontin is also expressed by a number of nonmineralizing tissues, as well as by transformed cells (24,25) and activated lymphocytes and macrophages (3,26), kidney epithelial cells, luminal epithelial cells of several organs (27) and smooth muscle cells in atherosclerotic lesions of the aorta (28). Osteocalcin is known to localize in bone, cementum, dentin and the formative cells of these hard tissues (28), but its function remains unclear, although it has been suggested to play a role in delaying nucleation and preventing excessive crystal growth (29).

It appears that Hertwig's epithelial root sheath cells are responsible for initiating the spatial and temporal differentiation of odontoblasts and cementoblasts into matrix-producing cells. The intermediate layer of cementum may be produced by Hertwig's epithelial root sheath cells (9–11,30–33). Moreover, Bosshardt *et al.* suggested that cementoblasts originate from the Hertwig's epithelial root sheath (8,32), but species differences make the origin and differentiation of cementoblasts inconsistent between different studies, and the understanding of cementogenesis is still incomplete.

Mouri *et al.* demonstrated expression of alkaline phosphatase and osteopontin mRNA in cultured epithelial cells from human periodontal ligament (12). Rincon *et al.* also demonstrated the production of osteopontin mRNA, but not of alkaline phosphatase mRNA, by cultured porcine epithelial rests of Malassez cells (34,35). Moreover, Hasegawa *et al.* reported that epithelial rests of Malassez cells are immunoreactive for osteopontin and ameloblastin during early cementum repair after experimental induction of root resorption *in vivo* (11). It is conceivable that epithelial rests of Malassez cells, as well as Hertwig's epithelial root sheath cells, may have a direct or indirect role in the formation of cementum. Immunohistochemistry, reverse transcription-polymerase chain reaction (RT-PCR) and *in situ* hybridization have been particularly useful approaches in the study of mineralized tissues, because they allow correlation of differentiated cell function with tissue organization and extracellular matrix assembly and structure during normal development, as well as during tissue repair after injury. The current study was undertaken to determine whether putative epithelial rests of Malassez cells can produce amelogenin, alkaline phosphatase and the noncollagenous proteins osteopontin, osteocalcin and bone sialoprotein, thereby supporting their involvement in the formation of cementum.

Material and methods

Cell culture

Fresh extracted third molars from 36 patients between 17 and 25 years of age were obtained from the Oral Surgery Department, Tohoku University Graduate School of Dentistry. Informed consent was obtained from the patients prior to extractions. The human ethics board of Tohoku University Graduate School of Dentistry specifically granted permission for our work with human subjects. After washing the teeth several times with α-minimum essential medium (Cosmo Bio Co. Ltd, Tokyo, Japan) supplemented with 10% fetal bovine serum and antibiotics (60 μg/mL of kanamycin, 20 units/mL of penicillin G, 10 μg/mL of Fungisone), human periodontal ligament explants attached to the mid-third of each root were carefully removed with a scalpel. The explants were plated onto 35-mm culture dishes using the supplemented α-minimum essential medium, and this procedure produced outgrowths that were primarily composed of periodontal ligament fibroblasts. After 1 wk, the cells were cultured in a modified serum-free medium [3 : 1, v/v; MCDB153 medium; Sigma Chemical Co., St. Louis, MO, USA) supplemented with 5 μg/mL of insulin (Sigma), 0.5 μg/mL of hydrocortisone (Sigma), 10 μg/mL of transferrin (Sigma), 14.1 μg/mL of phosphorylethanolamine (Sigma), 10 ng/mL of epidermal growth factor (Sigma) (36–38): α-minimum essential medium] including 40 μg/mL of bovine pituitary extract (Kyokuto, Tokyo, Japan) and antibiotics (39–42). This procedure resulted in outgrowths of epithelial cells as well as of fibroblasts. Cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Differential adhesion to the surface in the presence of 0.075 mg/mL of protease solution (Sigma) was used to produce cultures that were predominantly of one cell type [i.e. either fibroblasts (5–10 min) or epithelial cells (15–20 min)] from cultures that contained both cell types (43). Human gingival tissues were removed from clinically healthy patients at the time of the third molar extraction. After mincing, they were cultured as described above. Gingival epithelial cells and periodontal ligament fibroblasts cultured in a modified serum-free medium were used as controls.

Immunohistochemistry

The cells were fixed with 4% paraformaldehyde at room temperature for 10 min, and 3% hydrogen peroxide was used to inhibit endogenous peroxidase. After incubation with 5% normal goat serum for 30 min to block nonspecific binding, the cells were then treated at 4°C overnight with a primary antibody to monoclonal mouse antihuman cytokeratin AE1/AE3 (1 : 50) (Dako, Carpinteria, CA, USA) to confirm the presence of epithelial cells. Furthermore, the cells were incubated at 4°C overnight with primary antibodies to polyclonal rabbit antiporcine 25,000 kDa amelogenin

(0.1 μg/mL), of which the production and characterization have been described previously (1,2), monoclonal mouse antihuman cytokeratin 14 (1 : 100) (YLEM, Rome, Italy), monoclonal mouse antihuman osteopontin (0.1 μg/mL) (IBL, Gunma, Japan) and polyclonal rabbit antihuman osteocalcin (1 : 250) (Biomedical Technology Inc., Stoughton, MA, USA), and polyclonal rabbit antihuman bone sialoprotein (LF-84) (1 : 400) (courtesy of Dr L. W. Fisher, The National Institutes for Dental and Craniofacial Research, Bethesda, MD, USA) (44,45). The primary amelogenin antiserum used in this study can recognize both human and rat amelogenin (1,2). After rinsing in phosphate-buffered saline, the cells were incubated with biotinylated immunoglobulin at room temperature for 1 h and stained by the avidin-biotinylated peroxidase complex method, using an ExtrAvidin® peroxidase staining kit (Sigma) and an AEC (3-amino-9-ethylcarbazole) chromogen kit (Sigma). Mayer's hematoxylin solution was applied for counterstaining. Phosphate-buffered saline, instead of the primary antibody, and normal rabbit serum and phosphate-buffered saline, instead of the primary antiserum, were used for control staining.

Determination of alkaline phosphatase activity

The cells were fixed with 4% paraformaldehyde at room temperature for 10 min. To determine the localization of alkaline phosphatase in cultured cells, the cells were stained histochemically for alkaline phosphatase, according to a modified version of the Azo-dye coupling method (46).

In situ hybridization

The oligonucleotide probes used for the *in situ* hybridization were synthesized by Nihon Gene Research Laboratories Inc., Sendai, Japan. The sequences are shown in Table 1. Biotin was labeled at the 3′ end. A computer-assisted search (GenBank) of these antisense sequences, and of the corresponding sense sequences, revealed no significant homology with any known sequences other than the amelogenin and cytokeratin 14 sequences.

In situ hybridization was carried out using an *In Situ* Hybridization Detection Kit for Biotin-Labeled Probes (Sigma). Briefly, the cells were fixed with 4% paraformaldehyde at room temperature for 10 min. They were then immersed in phosphate-buffered saline (including RNase inhibitor) at room temperature, containing 3% hydrogen peroxide to inhibit endogenous peroxidase. The specimens were hybridized with biotin-labeled probes in the hybridization solution in a humid incubation chamber overnight at 37°C. After washing in phosphate-buffered saline, the specimens were reacted with blocking solution (phosphate-buffered saline containing 5% bovine serum albumin, 500 μg/mL of normal sheep immunoglobulin G, 100 μg/mL of salmon testicular DNA and 100 μg/mL of yeast tRNA) at room temperature for 15 min. After incubation with ExtrAvidin® peroxidase solution (Sigma) at 37°C for 20 min, the samples were reacted with biotin-conjugated antiavidin antibody (Sigma) in a humid chamber at room temperature for 30 min. They were then washed three times in phosphate-buffered saline. The peroxidase sites were visualized using a solution containing 3,3′-diaminobenzidine and hydrogen peroxide and then counterstained with Mayer's hematoxylin solution.

RT-PCR

Cells disrupted with RLT Buffer (a buffer containing guanidine thiocyanate and β-mercaptoethanol; Qiagen Pty Ltd, Victoria, Australia) were collected in microcentrifuge tubes. Total cellular RNA was isolated from cultured cells using an RNeasy® Mini Kit (Qiagen Pty Ltd), according to the manufacturer's instructions. A 0.05 μg/μL sample of total RNA was used as a template for RT-PCR. One-step RT-PCR was performed using a SuperScript™ one-step RT-PCR with a Platinum®Taq kit (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions, and a Programmable Thermal Controller PTC-100 (MJ Research, Watertown, MA, USA). To quantify the expression of alkaline phosphatase and noncollagenous bone proteins (osteopontin, osteocalcin and bone sialoprotein), semiquantitative RT-PCR, relative to glyceraldehyde-3-phosphate dehydrogenase, was performed. Amplimers designed for these four molecules and related information are provided in Table 2.

The thermal profile used for alkaline phosphatase, osteopontin, osteocalcin, bone sialoprotein and glyceraldehyde-3-phosphate dehydrogenase amplification was 40 cycles, starting with denaturation for 1 min at 94°C, followed by 1 min of annealing at 58°C and 1 min of extension at 72°C. The PCR products were subjected to electrophoresis, and digital images were obtained and analyzed using IMAGE J (NIH image) software. Three cell populations of each cell type were studied. Statistical analysis of the results was carried out using one-way analysis of variance, with p-values of < 0.01 considered significant.

Table 1. Oligonucleotide probes used for *in situ* hybridization. AME, amelogenin; CK14, cytokeratin 14

Oligo name	Sequence (5′ → 3′)	mer	Label	Reference
AME	CAT GGG TTC GTA ACC ATA GGA AGG	24	3′ Biotin	(47)
CK14	ACA TGA TGA CAT TCT TAG CCA CGT	24	3′ Biotin	(48)

Results

Immunohistochemistry

Putative epithelial rests of Malassez cells stained positive for broad-spectrum antibodies to cytokeratins (AE1/AE3), indicating their epithelial origin (Fig. 1A), but periodontal ligament fibroblasts did not show cytokeratin expression (Fig. 1B). Intense immunoreactivity for amelogenin was observed in putative epithelial rests of Malassez cells, whereas gingival epi-

Table 2. Oligonucleotide primers used for reverse transcription polymerase chain reaction

Gene (fragment)	Primer sequences	Denaturation/ annealing / extension (°C)	Cycle	Reference
ALPase (475 bp)	5′-ACGTGGCTAAGAATGTCATC-3′ 5′-CTGGTAGGCGATGTCCTT-3′	94/58/72	40	(49)
OPN (126 bp)	5′-CCAAGTAAGTCCAACGAAAG-3′ 5′-GGTGATGTCCTCGTCTGTA-3′	94/58/72	40	(50)
OC (315 bp)	5′-GGCAGCGAGGTAGTGAAGA-3′ 5′-CTGGAGAGGAGCAGAACTG-3′	94/58/72	40	(51)
BSP (248 bp)	5′-CAACAGCACAGAGGCAGAA-3′ 5′-CGTACTCCCCCTCGTATTC-3′	94/58/72	40	(52)
GAPDH (485 bp)	5′-TGTTTGTGATGGGTGTGAA-3′ 5′-ATGGGAGTTGCTGTTGAAG-3′	94/58/72	40	(53)

ALPase, alkaline phosphatase; BSP, bone sialoprotein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OC, osteocalcin; OPN, osteopontin.

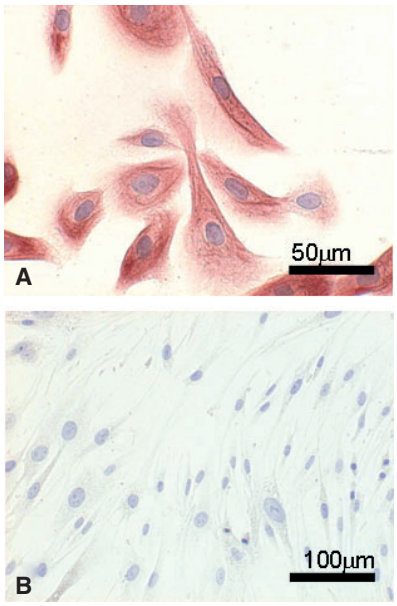

Fig. 1. Photomicrographs showing intense immunoreactivity for cytokeratin AE1/AE3 in the epithelial rests of malassez cells (E). Immunostaining For AE1/AE3 is not seen in fibroblasts from periodontal ligament cells (F).

thelial cells and periodontal ligament fibroblasts did not show amelogenin expression (Fig. 2A–C). Weak immunoreactivity for cytokeratin 14 was evident in putative epithelial rests of Malassez cells, indicating their epithelial basal layer origin, and intense immunoreactivity for cytokeratin 14 was found in gingival epithelial cells (Fig. 2G,H). Staining for cytokeratin 14 antibody was not detected in periodontal ligament fibroblasts (Fig. 2I), and hence immunohistochemical expression of cytokeratin 14, as well as AE1/AE3, was used to distinguish epithelial cells from fibroblasts. Although there is no morphological difference between putative epithelial rests of Malassez cells and gingival epithelial cells, these cells showed slight differences in the expression of cytokeratin 14.

Osteopontin was detected at high levels in putative epithelial rests of Malassez cells (Fig. 3D). Osteocalcin and bone sialoprotein proteins were not detected in putative epithelial rests of Malassez cells (Fig. 3G,J). Osteopontin, osteocalcin and bone sialoprotein proteins were detectable in periodontal ligament fibroblasts (Fig. 3F,I,L). As expected, noncollagenous bone proteins were not seen in gingival epithelial cells (Fig. 3E, H,K).

Determination of alkaline phosphatase activity

Putative epithelial rests of Malassez cells and periodontal ligament fibroblasts stained positively for alkaline phosphatase activity, but alkaline phosphatase-positive cells were not found in the cultures of gingival epithelial cells (Fig. 3A–C).

In situ hybridization to detect mRNAs for amelogenin and cytokeratin 14

To determine the localization of expression of mRNAs for amelogenin and cytokeratin 14, the cells were hybridized *in situ* with biotin-labeled antisense oligo-DNA probes. *In situ* hybridization showed considerable differences among putative epithelial rests of Malassez cells, gingival epithelial cells and periodontal ligament fibroblasts, and these differences are reflected by the immunohistochemical data. Hence, only putative epithelial rests of Malassez cells showed stronger positive signals for amelogenin (Fig. 2D). mRNA for cytokeratin 14 were detectable very weakly in putative epithelial rests of Malassez cells (Fig. 2J). The intense signal for cytokeratin 14 mRNA was detected in the cultures of gingival epithelial cells (Fig. 2K). Cytokeratin 14 mRNA expression was not detected in periodontal ligament fibroblasts, as expected (Fig. 2L).

RT-PCR

The expression of four different genes (mRNAs of alkaline phosphatase, osteopontin, osteocalcin and bone sialoprotein) was investigated in three cell populations – putative epithelial rests of Malassez cells, gingival epithelial cells and periodontal ligament fibroblasts – by using RT-PCR (Fig. 4). The relative intensities compared with glyceraldehyde-3-phosphate dehydrogenase are graphically illustrated in Fig. 5. The expression of mRNA for alkaline phosphatase and osteopontin was significantly higher in both putative epithelial rests of Malassez cells and periodontal ligament fibroblasts than in gingival epithelial cells ($p < 0.01$). Osteocalcin mRNA expression was weak in both putative epithelial rests of Malassez cells and periodontal ligament fibroblasts, and there was a very low level of expression in gingival epithelial cells; there were no significant differences in the relative intensities of osteocalcin mRNA among the three cell populations. Bone sialoprotein mRNA expression was at a very low level of expression in putative epithelial rests of Malassez cells and gingival epithelial cells compared with periodontal ligament fibroblasts ($p < 0.01$).

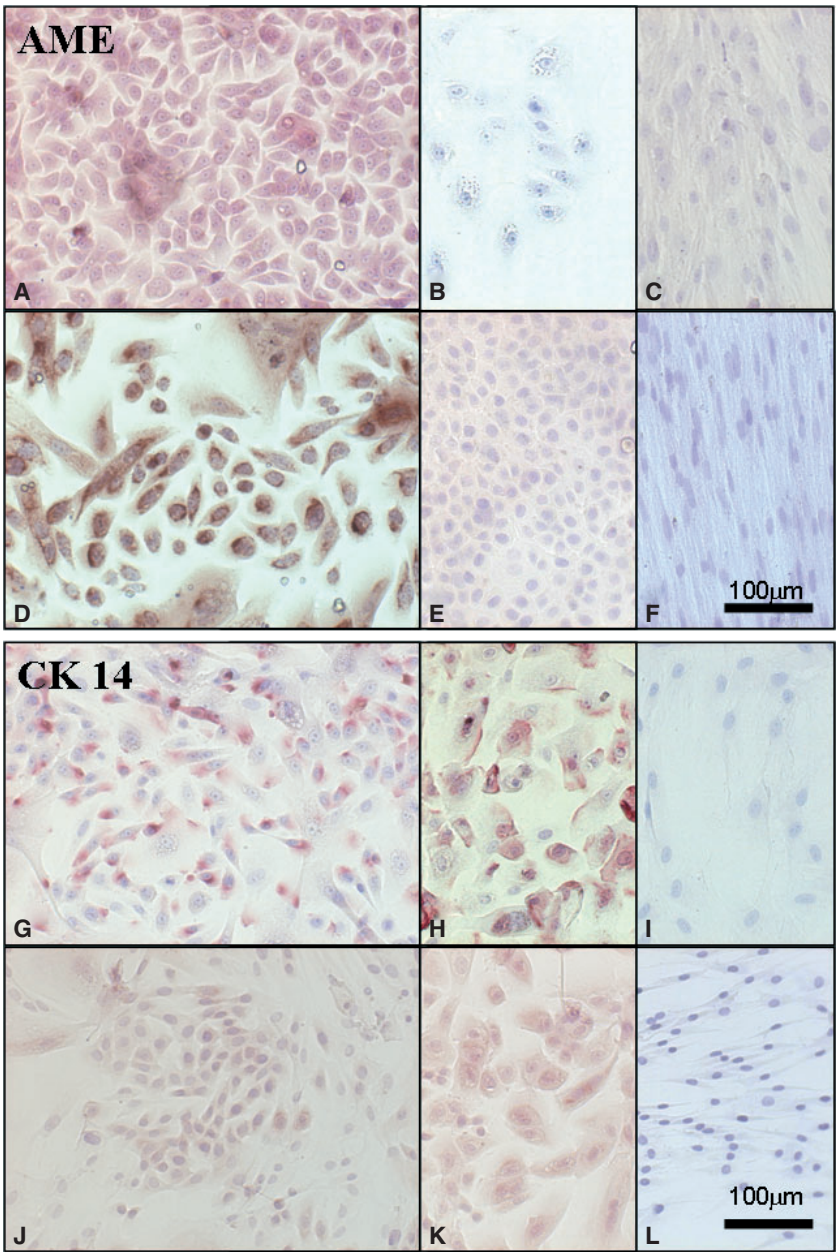

Fig. 2. Photomicrographs showing intense immunoreactivity for amelogenin in the epithelial rests of Malassez cells (A). Immunostaining for amelogenin is not seen in the gingival epithelial cells and the fibroblasts from periodontal ligament cells (B, C). Photomicrographs showing weak immunoreactivity for cytokeratin 14 in the epithelial rests of Malassez cells (D) and intense immunoreactivity for cytokeratin 14 in the gingival epithelial cells (E). Immunostainings for amelogenin and cytokeratin 14 are not seen in the fibroblasts from periodontal ligament cells (F). Photomicrographs showing intense immunoreactivity for amelogenin mRNA in the epithelial rests of Malassez cells (G). Immunostaining for amelogenin mRNA is not seen in the gingival epithelial cells and the fibroblasts from periodontal ligament cells (H, I). Photomicrographs showing weak immunoreactivity for cytokeratin 14 mRNA in the epithelial rests of Malassez cells (J) and intense immunoreactivity of cytokeratin 14 mRNA in the gingival epithelial cells (K). Immunostainings for amelogenin mRNA and cytokeratin 14 mRNA are not seen in the fibroblasts from periodontal ligaments cells (L). AME, ameliogenin; CK 14, cytokeratin 14.

Discussion

Following fenestration of Hertwig's epithelial root sheath cells, precementoblasts appear between the separated inner epithelial cells on the root surface, and the acellular cementum is formed by cementoblasts, a particular class of cells that resemble fibroblasts. However, using light and electron microscopy, Bosshardt & Schroeder showed that inner epithelial Hertwig's epithelial root sheath cells in human premolars resemble fibroblasts, and these cells have numerous cytoplasmic projections towards the predentin, and produce and attach the acellular cementum matrix to the as yet unmineralized dentinal matrix. Moreover, Bosshardt & Schroeder emphasized that this does not imply that the acellular cementum-producing cells are periodontal ligament fibroblasts or that they have the same origin as these cells, because such a morphological comparison might be misleading (32). We showed that elongated epithelial rests of Malassez cells that were morphologically similar to fibroblasts, but expressed cytokeratin AE1/AE3, were present in our culture system. Consistent with this, it is difficult to distinguish epithelial cells from fibroblasts morphologically.

Tabata *et al.* determined that cultured ameloblast-lineage cells are derived from the rat odontogenic epithelium, and that cytokeratin 14 is an earlier marker than amelogenin for ameloblast-lineage cells *in vitro* (13). Ravindranath *et al.* proposed that interactions between cytokeratin 14 and amelogenin may play an important role in amelogenesis, enamel development and disease, because cytokeratin 14 is specifically present in the basal layer of epidermis (14). Our data demonstrate that epithelial rests of Malassez cells express cytokeratin 14 at a lower level than human gingival epithelial cells. Considering that odontoblasts derived from ectomesenchymal cells also express amelogenin, we propose that both cytokeratin 14 and amelogenin are useful markers for cultured epithelial rests of Malassez cells, and that these cells are derived from the

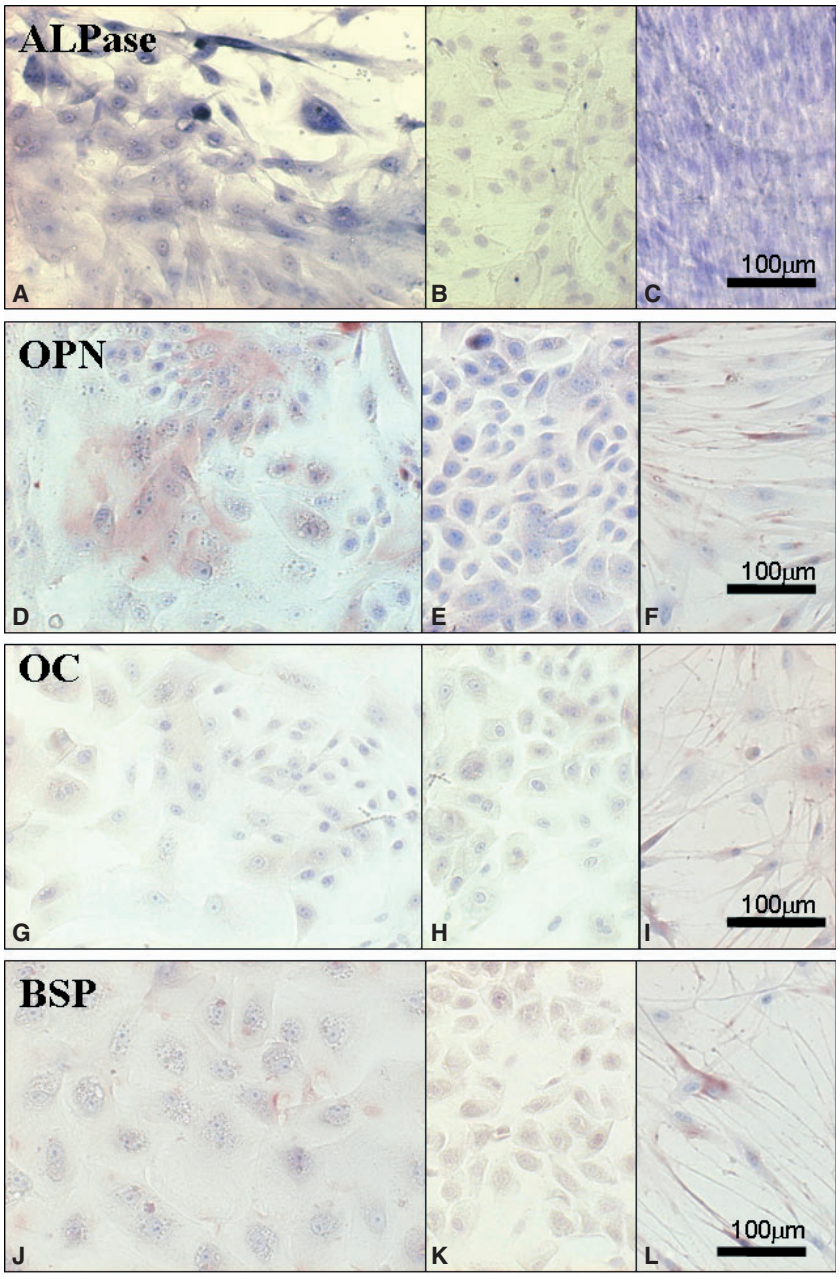

Fig. 3. Photomicrographs showing intense alkaline phosphatase activity in the epithelial rests of Malassez cells and the fibroblasts from periodontal ligament cells (A,C). No alkaline phosphatase activity is observed in the gingival epithelial cells (B). Photomicrographs showing immunoreactivity for osteopontin in the epithelial rests of Malassez cells (D) and for osteopontin, osteocalcin and bone sialoprotein in the fibroblasts from periodontal ligament cells (F, I, L). Immunostainings for osteocalcin and bone sialoprotein are not seen in the epithelial rests of Malassez cells (G, J). Immunostainings for osteopontin, osteocalcin and bone sialoprotein are not seen in the gingival epithelial cells (E, H, K). ALPase, alkaline phosphatase; BSP, bone sialoprotein; OC, osteocalcin; OPN, osteopontin.

odontogenic epithelium. In fact, in our experimental system, odontoblasts could not be cultured because the explants were extracted from adult periodontal ligament tissue after root formation, whereas amelogenin mRNA expression has been reported in odontoblasts (7).

The periodontium is a complex organ comprising gingival tissue, periodontal ligament, cementum and alveolar bone. Although it is widely held that cementoblasts represent a specialized type of cell, it is unclear where the cementoprogenitor cells originate from and what signals trigger their differentiation into cementoblasts. It is difficult to identify cementoblasts from periodontal ligament, because cementoblasts and periodontal ligament fibroblasts are thought to intermingle after the initial phase of acellular cementum formation. After root completion, the thickness of the acellular extrinsic fiber cementum along the root surface continues to increase slowly, and epithelial rests of Malassez cells still reside along the acellular cementum. *In situ* hybridization studies by Luo *et al*. indicate that enamel proteins expressed during root formation on developing mouse molars do not contain amelogenin (54). Moreover, Fong *et al*. demonstrated that epithelial cells located along the acellular cementum after its initial formation express neither amelin nor amelogenin, whereas amelin and amelogenin are found in epithelial cells enclosed at the border between cellular cementum and dentin (9). However, several recent studies have provided evidence that amelogenin also acts as a signal molecule during mineralized tissue formation. In rat molars with experimentally induced pulpal inflammation, Hamamoto *et al*. showed that amelogenin is formed by islands of epithelial cells at the apical end of the root (2). In immunohistochemical studies of developing human premolars, Hammarström showed that amelogenin is deposited on the dentin surface before formation of the acellular cementum. It should be pointed out that amelogenin is located in the cervical area of developing rat molars where the enamel matrix extends for a short distance between dentin and acellular cementum (10), and thus the expression of enamel proteins may be related to cementum formation during the development of the dental root. On the other hand, Bosshardt *et al*. suggest that Hertwig's epithelial root sheath cells occasionally assume a lingering ameloblastic activity at the beginning of root formation in the pig,

and they do not support the hypothesis of a causal relationship between enamel matrix proteins and cementogenesis (8). Moreover, they indicated that ectopic enamel deposits on the root retaining a high amount of amelogenin, whereas cementicles contain bone sialoprotein and osteopontin, typically found in bone and cementum (55). Hertwig's epithelial root sheath cells may have the possibility of both enamel formation and cementum formation, and they may participate in either enamel formation or cementum formation under different situations. A hypothesis advanced by Slavkin & Boyde proposes that Hertwig's epithelial root sheath cells synthesize proteins that are related to enamel polypeptides, and that these Hertwig's epithelial root sheath-derived proteins are instructive for ectomesenchymal determination, leading to cementoblast differentiation and acellular cementum (30). It has also been suggested that the intermediate layer of the cementum, seen in rodent molars, is produced by Hertwig's epithelial root sheath cells (9–11,30–33). The origin of cementogenesis is still controversial, but it appears that the extracellular matrix and the constructed cementum are formed by both epithelial rests of Malassez cells and periodontal ligament fibroblasts, although it is difficult to identify the role of cementoblasts with certainty.

In situ hybridization analyses to localize mRNAs for alkaline phosphatase and noncollagenous bone proteins also showed that there were considerable differences among putative epithelial rests of Malassez cells, human gingival epithelial cells and human periodontal ligament fibroblasts, and these were reflected in the immunohistochemical and histochemical data (data not shown). In our study, higher expression of amelogenin, alkaline phosphatase and osteopontin were found in putative epithelial rests of Malassez cells. Mouri *et al*. demonstrated expression of mRNA for alkaline phosphatase and osteopontin bone morphogenetic protein-2 and -4 in cultured human periodontal ligament epithelial cells (12). More recently, Rincon *et al*. also

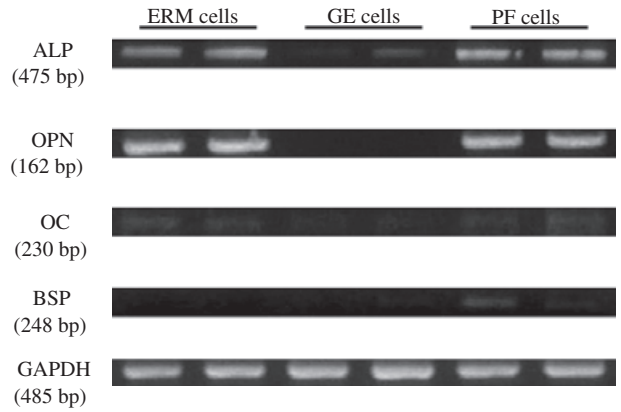

Fig. 4. Reverse transcription polymerase chain reaction products separated on 2% agarose gels and visualized via ethidium bromide. ALPase, alkaline phosphatase; BSP, bone sialoprotein; ERM, epithelial rests of Malassez; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GE, gingival epithelial; OC, osteocalcin; OPN, osteopontin; PF, periodontal ligament fibroblasts.

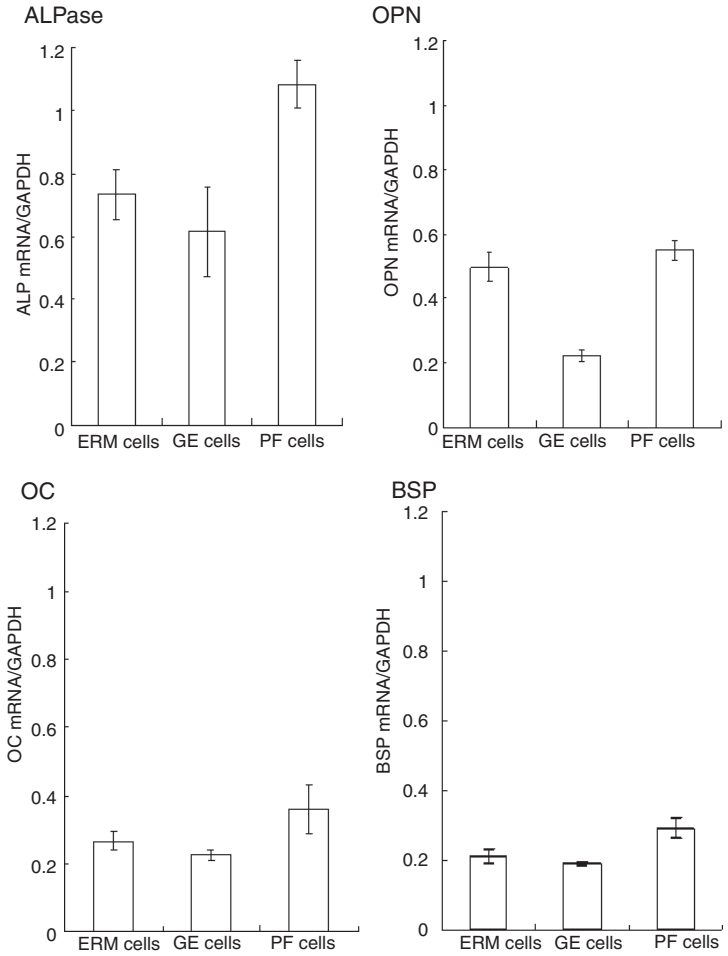

Fig. 5. Graphical representation of relative band intensities standardized by glyceraldehydes-3-phosphate dehydrogenase. ALPase, alkaline phosphatase; BSP, bone sialoprotein; ERM, epithelial rests of Malassez; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GE, gingival epithelial; OC, osteocalcin; OPN, osteopontin; PF, periodontal ligament fibroblasts.

demonstrated production of osteopontin mRNA, but not of mRNAs for other related proteins, by cultured porcine epithelial rests of Malassez cells (34,35). On the other hand, osteocalcin protein was not detected, and osteocalcin mRNA was detectable only very weakly, in putative epithelial rests of Malassez cells. Bone sialoprotein protein and its corresponding mRNA were not detected in putative epithelial rests of Malassez cells. Ivanonski *et al.* demonstrated that osteopontin and osteocalcin are present in normal human periodontal ligament tissue, whereas bone sialoprotein is found in cementum and alveolar bone (49). As bone sialoprotein was abundant in the more mature osteoblast cell populations and appeared to be related to mineralization potential, it may be a more reliable marker of the later development stage. Putative epithelial rests of Malassez cells have the ability to express some of the bone-associated proteins, but may not transform the maturing cells in our culture system.

Noncollagenous bone proteins are considered to have multiple functions related to the formation, turnover and repair of collagen-based mineralized tissues. Previous studies have shown that these proteins are present in periodontal ligament, mantle dentin, acellular and cellular cementum, and cementoblasts (33,49,56–58). Periodontal ligament tissue contains mixtures of cementoblast, fibroblast and osteoblast precursor cells, as well as epithelial rests of Malassez cells. We also showed that cultured periodontal ligament fibroblasts express osteopontin, osteocalcin and bone sialoprotein proteins and their corresponding mRNAs, and have strong alkaline phosphatase activity and alkaline phosphatase mRNA expression; these findings were based on a sensitive detection method using signal amplification of complexes by ExtrAvidin®. Periodontal ligament fibroblasts may also have the potential to regulate hard tissue formation. As fibroblasts adjacent to the hard tissue facilitate tissue biomineralization, while fibroblasts distal to the hard tissue inhibit this process in normal periodontal ligament, the interaction between fibroblasts and epithelial rests of Malassez cells, or between fibroblasts and osteoblasts, may facilitate the potential activity of biomineralization.

Nohutcu *et al.* reported that the expression of bone sialoprotein and osteopontin mRNAs was detectable in cells obtained from freshly isolated porcine periodontal ligament tissues, but occurred at a much lower level in cultured periodontal ligament cells, while there was an ≈three-fold increase in alkaline phosphatase mRNA expression in cultured cells compared with fresh periodontal ligament tissue (59). Cell culture favors growth and selects for the most rapidly proliferating cells, and therefore cell culture data should be cautiously extrapolated to the *in vivo* situation, particularly as evidence for cellular origin. However, valuable information can still be obtained from *in vitro* studies, because the results reflect various situations *in vivo*. Moreover, cells derived from periodontal ligament should produce the same proteins and mRNAs expressed in periodontal ligament tissues.

In conclusion, cultured epithelial rests of Malassez cells expressed amelogenin, alkaline phosphatase and osteopontin, suggesting that epithelial rests of Malassez cells may participate in cementum formation, but they did not enhance osteocalcin and bone sialoprotein expression.

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