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Mutual induction of noncollagenous bone proteins at the interface between epithelial cells and fibroblasts from human periodontal ligament

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Background and Objective: Epithelial–mesenchymal interactions are responsible for cell differentiation during periodontal regeneration. The present study was undertaken to examine the expression of alkaline phosphatase and noncollagenous bone proteins, such as osteopontin, osteocalcin and bone sialoprotein, with respect to interaction between the cells of the epithelial rests of Malassez and fibroblasts from human periodontal ligament.

Material and Methods: Explants of human periodontal ligament tissues produced outgrowths containing both putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts in a modified serum-free medium. Putative epithelial rests of Malassez cells cultured alone, and human periodontal ligament fibroblasts cultured alone, were used as controls. The expression levels of amelogenin were analyzed by *in situ* hybridization. The expression and distribution of alkaline phosphatase and noncollagenous bone proteins in both cell populations at the interface between putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts were analyzed by immunohistochemistry, *in situ* hybridization and reverse transcription-polymerase chain reaction.

Results: Amelogenin mRNA was detected at high levels only in putative epithelial rests of Malassez cells at the interface. Alkaline phosphatase and bone sialoprotein mRNAs were detected significantly at the interface between putative epithelial rests of Malassez cells and human periodontal ligament fibroblast cells. In particular, bone sialoprotein and its mRNA were expressed significantly in human periodontal ligament fibroblasts at the interface between putative epithelial rests of Malassez cells and human periodontal ligament fibroblast cells. The expressions of osteopontin and its mRNA were not different between putative epithelial rests of malassez cells and human periodontal ligament fibroblasts at the interface. Osteocalcin and its mRNA were expressed strongly in putative epithelial rests of

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Malassez cells at the interface between putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts.

Conclusion: These findings indicate that the epithelial–mesenchymal interaction modulates the expression of alkaline phosphatase, osteocalcin and bone sialoprotein in putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts, suggesting that epithelial–mesenchymal interactions play a role in the maintenance of periodontal ligament.

Human periodontal ligament contains the epithelial rests of Malassez, which appear as cellular clusters in cord- or net-like formations or as isolated islands near the cementum. Hertwig's epithelial root sheath cells represent an extension of the epithelium that participates in coronal amelogenesis and which is retained in association with the developing root. Immediately as the outer layer of the dentin matrix starts to calcify, the epithelial cells of the root sheath separate from the dentin surface, and breaks occur in the continuity of its previously continuous double-layered sheet of cells to form the epithelial rests of Malassez. As a result, the epithelial rests of Malassez are odontogenic epithelial cells derived from Hertwig's epithelial root sheath. Amelogenin is the major enamel protein produced by ameloblasts at the differentiation and secretory stages (1). We demonstrated previously that intense immunoreactivity for amelogenin mRNA is observed in putative epithelial rests of Malassez cells, whereas gingival epithelial cells and human periodontal ligament fibroblasts do not show amelogenin mRNA expression (2). Amelogenin is therefore a useful marker for cultured epithelial rests of Malassez cells.

The formation, turnover and repair of mineralized tissues, such as bone, calcified cartilage and teeth, are all complex processes requiring differentiated cell function and extracellular matrix assembly and degradation during normal development as well as during tissue repair after injury. It has been determined that several of the mineral-binding proteins are capable not only of guiding extracellular calcification, but also of mediating cell dynamics, such as cell adhesion, spreading and migration, in mineralized tissues (3–5). 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 (6–9). Osteopontin, osteocalcin and bone sialoprotein are the major noncollagenous proteins that are secreted by osteoblastic cells and deposited into the bone matrix (5). Osteopontin is highly phosphorylated and sulfated but is unlikely to be a primary nucleator of hydroxyapatite formation because it was found to appear before bone sialoprotein as a marker of an early stage of bone formation during the regenerative processes (10). Moreover, it has been demonstrated that osteopontin inhibits mineralization (3,10–13). Indeed, osteopontin is also expressed by a number of nonmineralizing tissues, as well as by transformed cells (14,15) and activated lymphocytes and macrophages (3,16), kidney epithelial cells, luminal epithelial cells of several organs (17) and smooth muscle cells in atherosclerotic lesions of the aorta (18). Osteocalcin is known to localize in bone, cementum and dentin, and in the formative cells of these hard tissues (5,18). Its function, however, is still not clear, although it has been implicated to play a role in delaying nucleation and in preventing excessive crystal growth (19). Bone sialoprotein appears to be unique to mineralized connective tissue. In addition, bone sialoprotein has the potential to mediate the initial formation of hydroxyapatite crystals (20–22).

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 (23–29). Moreover, Bosshardt *et al.* suggested that cementoblasts originate from Hertwig's epithelial root sheath (29,30), but species differences make the origin and differentiation of cementoblasts inconsistent between different studies, and therefore the understanding of cementogenesis is still incomplete. Mouri *et al.* demonstrated expression of alkaline phosphatase and osteopontin mRNA in cultured epithelial rests of Malassez cells from human periodontal ligament (31). Rincon *et al.* also demonstrated the production of osteopontin mRNA, but not of alkaline phosphatase mRNA, by cultured porcine epithelial rests of Malassez cells (32,33). Moreover, Hasegawa *et al.* reported that epithelial rests of Malassez cells are immunoreactive for osteopontin and ameloblastin during early cementum repair after the experimental induction of root resorption *in vivo* (26). 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.

Some authors have suggested that epithelial rests of Malassez play a role in the maintenance of the periodontal space. Epithelial rests of Malassez cells were found to be stimulated to a more proliferating state as a result of orthodontic tooth movement, inflammatory conditions, or wound healing (34–39). A regrowth of epithelial rests of Malassez cells may be linked to reconstitution of the periodontal ligament and repair of the resorptive defect in the tooth surface. We demonstrated

previously that epithelial rests of Malassez cells have a high proliferation rate at the interface between epithelial rests of Malassez cells and human periodontal ligament fibroblasts and that the synthesis of type IV collagen and laminin is induced by direct interaction between epithelial rests of Malassez cells and human periodontal ligament fibroblasts (40). Considering the homeostasis of the periodontium, much seems to need to be done regarding the aspects of epithelial–mesenchymal interactions.

The present study was undertaken to determine whether epithelial rests of Malassez cells and human periodontal ligament fibroblasts at the interface *in vitro* have the ability to regulate alkaline phosphatase and the noncollagenous proteins, osteopontin, osteocalcin and bone sialoprotein, supporting their involvement in the maintenance of periodontal ligament.

Material and methods

Cell culture

Freshly extracted third molars from 24 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 project to work with human subjects. After washing the teeth several times with alpha minimal 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 amphotericin B), human periodontal ligament explants attached to the mid-third of each root were removed carefully from the root with a scalpel. The explants were plated into 35-mm culture dishes using the supplemented alpha minimal essential medium and produced outgrowths that were primarily composed of fibroblasts. After 1 wk the explants 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 transferrine (Sigma), 14.1 μg/mL of phosphorylethanolamine (Sigma), 10 ng/mL of epidermal growth factor (Sigma): alpha minimal essential medium (COSMO BIO Co. Ltd) including 40 μg/mL of bovine pituitary extract (Kyokuto, Tokyo, Japan) and antibiotics (40). Cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. This resulted in outgrowths of epithelial cells as well as of fibroblasts.

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 (41). Putative epithelial rests of Malassez cells cultured alone, and human periodontal ligament fibroblasts cultured alone, were used as controls.

Immunohistochemistry

The cells were fixed with 4% paraformaldehyde at room temperature for 10 min, and 3% H_2O_2 was used to inhibit endogenous peroxidase. The cells were then incubated with 5% normal goat serum for 30 min to block nonspecific binding, after which they were reacted at 4°C overnight with monoclonal mouse antihuman cytokeratin AE1/AE3 (1 : 50) (Dako, Carpinteria, CA, USA) to confirm the presence of epithelial cells. In addition, the cells were incubated at 4°C overnight with primary antibodies to monoclonal mouse antihuman osteopontin (0.1 μg/mL) (IBL, Gunma, Japan), 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, National Institutes for Dental and Craniofacial Research, Bethesda, MD, USA) (42–45). After rinsing in phosphate-buffered saline, the cells were incubated with biotinylated immunoglobulin at room temperature for 30 min and stained by the avidin-biotinylated peroxidase complex using an ExtrAvidin® peroxidase staining kit (Sigma) and the 3-amino-9-ethylcarbazole chromogen kit (Sigma). Mayer's hematoxylin solution was applied for counterstaining. Phosphate-buffered saline, instead of the primary antibody, and rabbit serum and phosphate-buffered saline, instead of the primary antiserum, were used for control staining.

Proliferating, DNA-synthesizing cells in cocultures were identified by nuclear incorporation of 100 μmol/L of 5-bromo-2′-deoxyuridine for 24 h. Labeled nuclei were detected with monoclonal mouse antibody to 5-bromo-2′-deoxyuridine (Boehringer Mannheim Biochemica, Mannheim, Germany) for 30 min at 37°C. After washing three times in phosphate-buffered saline, the cells were incubated with horseradish peroxidase-conjugated goat antimouse immunoglobulin (Chemicon International Inc., Temecula, CA, USA) for 30 min at 37°C and stained with 3,3′-diaminobenzidine and H_2O_2.

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 method 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. A biotin label was added at the 3′ end. A computer-assisted search (GenBank) of the antisense sequences shown in Table 1, as well as that of sense sequences, revealed no significant homology with any known sequences other than those of the amelogenin chain, the alkaline phosphatase chain,

Table 1. Oligonucleotide probes used for the in situ hybridization

Oligo name	Sequence (5′–3′)	Mer	Label	Reference
Amelogenin	CAT GGG TTC GTA ACC ATA GGA AGG	24	3′ Biotin	(47)
Alkaline phosphatase	ACA TGA TGA CAT TCT TAG CCA CGT	24	3′ Biotin	(48)
Osteopontin	ATG GCT TTC GTT GGA CTT ACT TGG	24	3′ Biotin	(49)
Osteocalcin	GCG AGG AGT GTG TGA GGG CTC ATG	24	3′ Biotin	(50)
Bone sialoprotein	ATT TTT CAT TGA GAA AGC ACA GGC	24	3′ Biotin	(51)

the osteopontin chain, the osteocalcin chain and the bone sialoprotein chain.

In situ hybridization was carried out using the *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 containing RNase inhibitor at room temperature and 3% H_2O_2 was used to inhibit endogenous peroxidase. The specimens were hybridized with biotin-labeled probes, present in the hybridization solution, in the humid incubation chamber overnight at 37°C. After washing in phosphate-buffered saline, the specimens were reacted with blocking solution (5% bovine serum albumin, 500 μg/mL of normal sheep IgG, 100 μg/mL of salmon testicular DNA and 100 μg/mL of yeast tRNA in phosphate-buffered saline) at room temperature for 15 min. The cells were then incubated with ExtrAvidin® peroxidase solution (Sigma) at 37°C for 20 min and reacted with biotin-conjugated anti-avidin immunoglobulin (Sigma) in a humid chamber at 37°C for 30 min. After washing three times in phosphate-buffered saline, the sites of peroxidase activity were visualized using a solution containing 3,3′-diaminobenzidine and H_2O_2 and counterstained by Mayer's hematoxylin solution. Sense oligonucleotide probes were used for control staining.

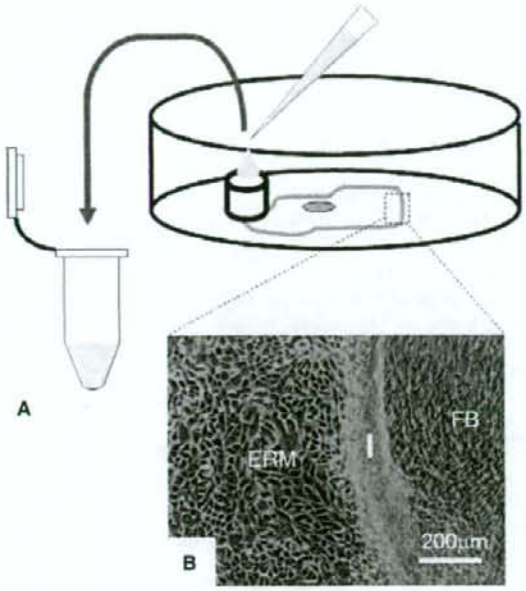

Fig. 1. (A) Schematic diagram showing the preparation of the samples at the interface between putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts, except for only putative epithelial rests of Malassez cells or only human periodontal ligament fibroblasts away from the interface in the same dishes. The silicone cylinders (5 mm in diameter) pasted with white petrolatum on the bottom, were placed on the dishes. (B) Phase-contrast photomicrograph taken of primary cultured putative epithelial rests of Malassez cells (ERM) and of human periodontal ligament fibroblasts (FB) at the interface (I).

Reverse transcription polymerase chain reaction (RT-PCR)

The interface area cells, which are present between putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts, were obtained using silicone cylinders (Fig. 1A,B) Briefly, silicone cylinders (5 mm in diameter) pasted with white petrolatum on the bottom, were placed on the dishes after aspirating the medium and washing twice with phosphate-buffered saline. Fifty microlitres of Buffer RLT (buffer containing guanidine thiocyanate and β-mercaptoethanol) (Qiagen Pty Ltd, Victoria, Australia) was added to the silicone cylinders. The cells disrupted with Buffer RLT were collected in the microcentrifuge tubes. Putative epithelial rests of Malassez cells cultured alone and human periodontal ligament fibroblasts cultured alone were sampled as controls. Total cellular RNA was isolated from cultured cells, according to the manufacturer's instructions, using an RNeasy® Mini Kit (Qiagen Pty Ltd), and 0.05 μg/μL of total RNA was used as a template for RT-PCR. One-step RT-PCR was performed using SuperScript™ one-step RT-PCR with the Platinum® Taq kit (Invitrogen Corp., 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 alkaline phosphatase, osteopontin, osteocalcin,

Table 2. Primer pairs used for reverse transcription-polymerase chain reaction amplification

Gene (fragment)	Printer sequences	Denaturation/annealing/ extension (°C)	Cycle	Reference
Alkaline phosphatase (475 bp)	5'-ACGTGGCTAAGAATGTCATC-3' 5'-CTGGTAGGCGATGTCCTTA-3'	94/58/72	40	(52)
Osteopontin (126 bp)	5'CCAAGTAAGTCCAACGAAAG-3' 5'-GGTGATGTCCTCGTCTGTA-3'	94/58/72	40	(53)
Osteocalcin (230 bp)	5'-GGCAGCGAGGTAGTGAAGA-3' 5'-CTGGAGAGGAGCAGAACTG-3'	94/58/72	40	(54)
Bone sialoprotein (248 bp)	5'-CAACAGCACAGAGGCAGAA-3' 5'-CGTACTCCCCCTCGTATTC-3'	94/58/72	40	(51)
GAPDH (485 bp)	5'-TGTTTGTGATGGGTGTGAA-3' 5-ATGGGAGTTGCTGTTGAAG-3'	94/58/72	40	(55)

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

bone sialoprotein and glyceraldehyde-3-phosphate dehydrogenase, and related information, are provided in Table 2. The thermal profile for human 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 with the use of IMAGE J (NIH image software, Bethesda, MD, USA). The data were consistent, as confirmed by three independent experiments. Statistical analysis of the results was carried out using the Bonferroni/Dunn post-hoc test, and p-values of < 0.05 were considered significant.

Results

Immunohistochemistry and determination of alkaline phosphatase activity

Putative epithelial rests of Malassez cells stained positive for broad-spectrum antibodies to cytokeratins (AE1/AE3), indicating their epithelial origin, whereas human periodontal ligament fibroblasts did not show cytokeratin expression at the interface in the same dishes (Fig. 2A). Putative epithelial rests of Malassez cells incorporated 5-bromo-2'-deoxyuridine more extensively than human periodontal ligament fibroblasts derived from the same periodontal ligament explant, indicating that putative epithelial rests of Malassez cells have a higher proliferation rate than human periodontal ligament fibroblasts (Fig. 2C).

Alkaline phosphatase activity was expressed strongly at the interface (Fig. 3A). Putative epithelial rests of Malassez cells cultured alone and human periodontal ligament fibroblasts cultured alone also stained strongly for alkaline phosphatase activity (Fig. 3B,C). Intense immunoreactivity for osteopontin was observed in both putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts at the interface (Fig. 3D). Osteopontin was also detected in putative epithelial rests of Malassez cells cultured alone and in human periodontal ligament fibroblasts cultured alone (Fig. 3E,F). Strong immunoreactivity for osteocalcin was observed in putative epithelial rests of Malassez cells but not in human periodontal ligament fibroblasts at the interface (Fig. 3G). In contrast, bone sialoprotein was detectable in human periodontal ligament fibroblasts but not in putative epithelial rests of Malassez cells at the interface (Fig. 3J). Osteocalcin and bone sialoprotein proteins were not detected in putative epithelial rests of Malassez cells cultured alone (Fig. 3H,K), but were present in human periodontal ligament fibroblasts cultured alone (Fig. 3I,L).

In situ hybridization to localize mRNAs for amelogenin, alkaline phosphatase and noncollagenous bone proteins

To localize the mRNA expression of amelogenin, alkaline phosphatase and noncollagenous bone proteins, the cells were hybridized *in situ* with biotin-labeled antisense oligo-DNA probes. Putative epithelial rests of Malassez cells showed strongly positive signals for amelogenin mRNA. However, the amelogenin mRNA signal was not detectable in human periodontal ligament fibroblasts (Fig. 2B). These results supported that putative epithelial rests of Malassez cells are different from human periodontal ligament fibroblasts and are derived from the odontogenic epithelial origin.

In situ hybridization analyses also showed considerable differences between putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts and reflected the histochemical and immunohistochemical data. The mRNAs of alkaline phosphatase, osteopontin and osteocalcin were expressed strongly in putative epithelial rests of Malassez cells at the interface, whereas bone sialoprotein mRNA expression was not detectable (Fig. 4A,D,G,J). On the other hand, mRNAs of alkaline phosphatase, osteopontin and bone sialoprotein were expressed strong in human periodontal ligament fibroblasts at the interface, whereas osteocalcin mRNA expression was not detectable (Fig. 4A,D,G,J). Putative epithelial rests of Malassez cells cultured alone showed strong positive signals for alkaline phosphatase and osteopontin mRNAs (Fig. 4B,E). mRNA for osteocalcin was detectable only very weakly (Fig. 4H) and mRNA for bone sialoprotein was not expressed at all in putative

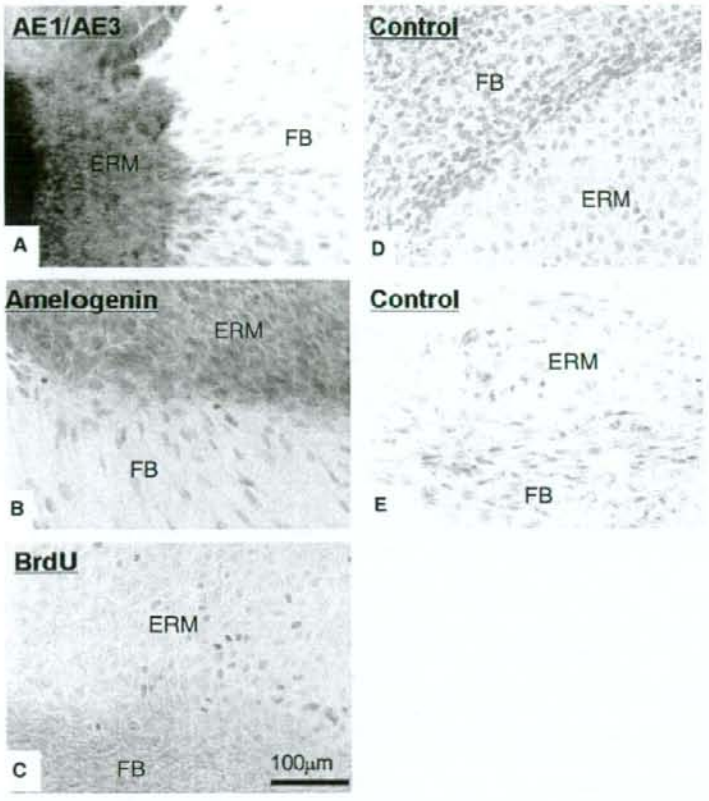

Fig. 2. (A) Photomicrograph showing strong immunoreactivity for cytokeratin AE1/AE3 in putative epithelial rests of Malassez cells. Immunostaining for cytokeratin in human periodontal ligament fibroblasts is not present. (B) Photomicrograph showing strong immunoreactivity for amelogenin mRNA in putative epithelial rests of Malassez cells. Immunostaining for amelogenin mRNA in human periodontal ligament fibroblasts is not present. (C) Photomicrograph showing assessment of proliferation by 5-bromo-2′-deoxyuridine labeling in putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts. (D) Photomicrograph showing negative-control immunostaining in putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts. (E) Photomicrograph showing the negative control hybridized *in situ* with biotin-labeled sense oligoDNA probes for amelogenin mRNA in putative epithelial rests of Malassez cells and in human periodontal ligament fibroblasts. ERM, epithelial rests of Malassez cells; FB, human periodontal ligament fibroblasts.

epithelial rests of Malassez cells cultured alone (Fig. 4K). On the other hand, mRNAs for osteopontin, osteocalcin and bone sialoprotein were detectable in human periodontal ligament fibroblasts cultured alone (Fig. 4F,I,L), and a strong signal for alkaline phosphatase mRNA was detected in human periodontal ligament fibroblasts cultured alone (Fig. 4C).

RT-PCR

The expression of four genes (mRNAs of alkaline phosphatase, osteopontin, osteocalcin and bone sialoprotein) was investigated in three cell populations, both putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts at the interface, putative epithelial rests of Malassez cells cultured alone and human periodontal ligament fibroblasts cultured alone, using RT-PCR (Fig. 5). The mRNA intensities relative to that of glyceraldehyde-3-phosphate dehydrogenase are presented in Fig. 6. The expression of mRNAs for alkaline phosphatase and bone sialoprotein were significantly stronger at the interface between putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts ($p < 0.05$). On the other hand, there were no significant differences in the relative intensities of mRNAs for osteopontin and osteocalcin among the three cell populations. The mRNA for osteocalcin was detectable only very weakly, and the mRNA for bone sialoprotein was not expressed at all in putative epithelial rests of Malassez cells cultured alone. A strong signal for alkaline phosphatase mRNA expression was detected in human periodontal ligament fibroblasts cultured alone, and mRNAs for osteopontin, osteocalcin and bone sialoprotein were detectable in human periodontal ligament fibroblasts cultured alone.

Discussion

Amelogenin is the major enamel protein produced by ameloblasts at the differentiation and secretory stages (3). Amelogenin has been observed to be localized in the enamel matrix, in normal ameloblasts, in odontogenic tumor cells, in Hertwig's epithelial root sheath cells and their extracellular matrices *in vivo*, in mantle dentin and in odontoblasts (3,30,56–60). In the present study, amelogenin mRNA expression distinguished putative epithelial rests of Malassez cells from human periodontal ligament fibroblasts at the interface, and putative epithelial rests of Malassez cells were the odontogenic epithelial cells derived from Hertwig's epithelial root sheath (Fig. 2B). Boshardt *et al.* suggest that Hertwig's epithelial root sheath cells occasionally assume a lingering ameloblastic activity at the start of root formation in the pig, but they do not support the hypothesis of a causal relationship between enamel matrix proteins and cementogenesis (30). Moreover, they indicated that ectopic enamel deposits

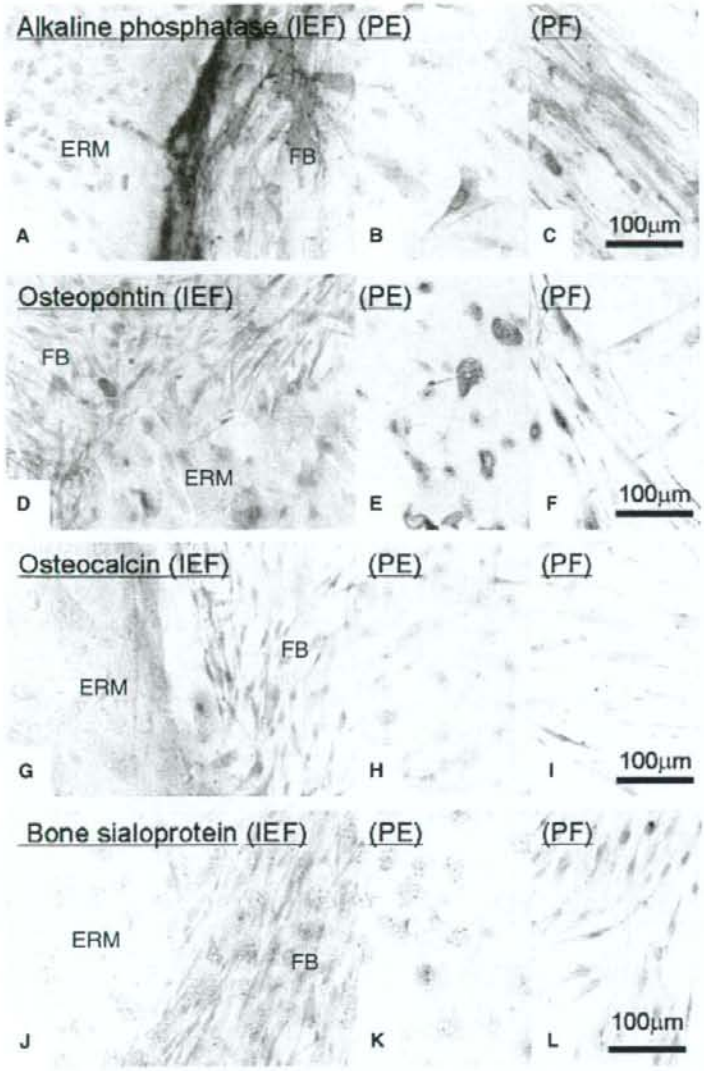

Fig. 3. (A–C) Photomicrographs showing strong alkaline phosphatase activity in interface area cells, in putative epithelial rests of Malassez cells cultured alone and in putative human periodontal ligament fibroblasts cultured alone. (D–F) Photomicrographs showing strong immunoreactivity for osteopontin in interface area cells. Osteopontin was also detected in putative epithelial rests of Malassez cells cultured alone and in putative human periodontal ligament fibroblasts cultured alone. (G–I) Photomicrographs showing strong immunoreactivity for osteocalcin in putative epithelial rests of Malassez cells at the interface area but very weak immunoreactivity for osteocalcin in putative human periodontal ligament fibroblasts cultured alone. Osteocalcin was not detected in putative epithelial rests of Malassez cells cultured alone. (J–L) Photomicrographs showing no immunoreactivity for bone sialoprotein in putative epithelial rests of Malassez of the interface area and in putative epithelial rests of Malassez cells cultured alone, and weak immunoreactivity for bone sialoprotein in human periodontal ligament fibroblasts of the interface area and putative human periodontal ligament fibroblasts cultured alone. ERM, epithelial rests of Malassez cells; FB, human periodontal ligament fibroblasts; IEF, interface area; PE, putative epithelial rests of Malassez cells cultured alone; PF, putative human periodontal ligament fibroblasts cultured alone.

on the root retain a high amount of amelogenin, whereas cementicles contain bone sialoprotein and osteopontin, typically found in bone and cementum (61). Hertwig's epithelial root sheath cells may be involved in both enamel formation and cementum formation, and they may participate in either enamel formation or cementum formation in different situations.

It has been suggested that epithelial rests of Malassez may protect the root surface from resorption, prevent ankylosis and consequently maintain the integrity of the periodontal ligament. Some studies have postulated that proliferating epithelial rests of Malassez cells may be involved in reconstitution of the periodontium (38,39,62). We showed, in the present study, that putative epithelial rests of Malassez cells have a higher proliferation rate, according to the incorporation of 5-bromo-2'-deoxyuridine extensively at the interface (Fig. 2C). Moreover, in our previous study, the synthesis of type IV collagen and laminin including the basement membrane was induced by the interaction at the interface between putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts (40). The initiation of cementogenesis is at least temporally linked to the disintegration process of Hertwig's epithelial root sheath. It is natural that the basement membrane is broken down in this disintegration. The disruption of a basement membrane may be correlated to the expression of osteopontin and bone sialoprotein. Osteopontin and bone sialoprotein are members of the small integrin-binding ligand, N-linked glycoprotein (SIBLING) family. The SIBLING family can specifically bind pro-matrix metalloproteinases (pro-MMPs) and activate MMPs. MMPs play a crucial role in extracellular matrix degradation during tissue remodeling. Moreover, epithelial growth *in vitro* and during wound healing *in vivo* is intrinsically linked to the expression of MMPs (63,64).

Alkaline phosphatase and noncollagenous bone proteins are considered to have multiple functions related to the formation, turnover and repair of collagen-based mineralized tissues. Previ-

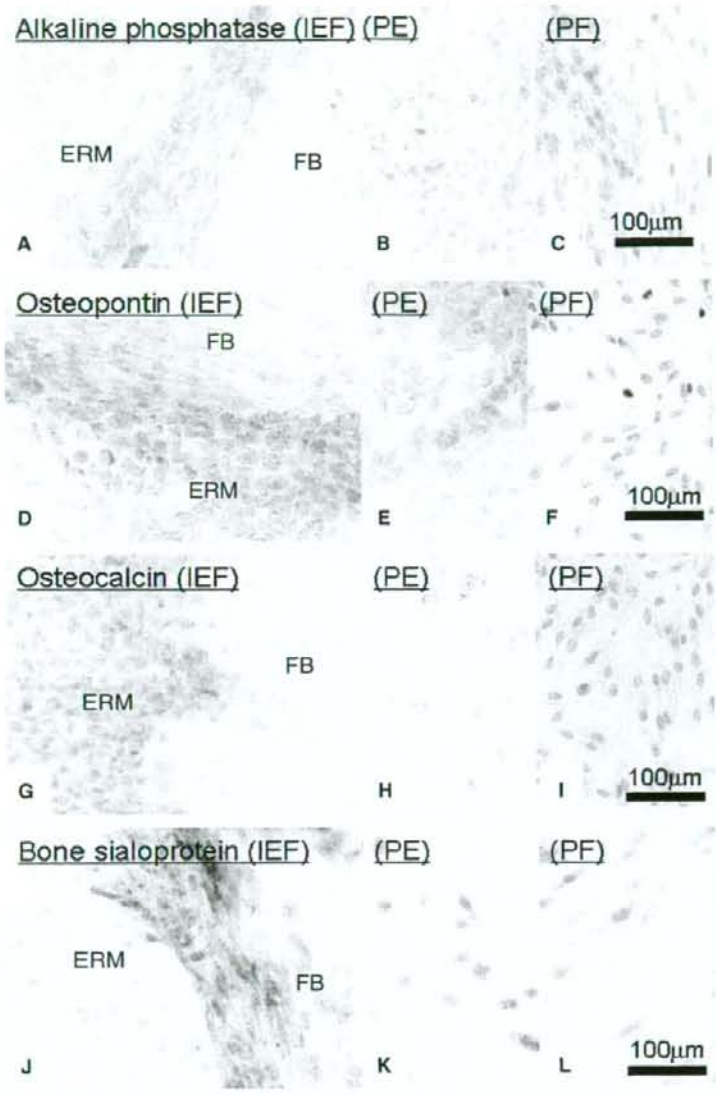

Fig. 4. (A–C) Photomicrographs showing strong immunoreactivity for alkaline phosphatase mRNA in interface area cells, in putative epithelial rests of Malassez cells cultured alone and in putative human periodontal ligament fibroblasts cultured alone. (D–F) Photomicrographs showing strong immunoreactivity for osteopontin mRNA in interface area cells. Osteopontin mRNA was also detected in putative epithelial rests of Malassez cells cultured alone and in putative human periodontal ligament fibroblasts cultured alone. (G–I) Photomicrographs showing strong immunoreactivity for osteocalcin mRNA in putative epithelial rests of Malassez cells of the interface area but very weak immunoreactivity for ostocalcin mRNA in putative human periodontal ligament fibroblasts cultured alone. Osteocalcin mRNA was not detected in putative epithelial rests of Malassez cells cultured alone. (J–L) Photomicrographs showing no immunoreactivity for bone sialoprotein mRNA in putative epithelial rests of Malassez cells of the interface area and in putative epithelial rests of Malassez cells cultured alone, but weak immunoreactivity for bone sialoprotein mRNA in human periodontal ligament fibroblasts of the interface area and in putative human periodontal ligament fibroblasts cultured alone.

ous studies have shown that alkaline phosphatase and noncollagenous bone proteins are present in periodontal ligament, mantle dentin, acellular and cellular cementum, and cementoblasts (10,20,28,52,65). An understanding of the distribution of these proteins within the human periodontium is of fundamental importance. When we set out to determine the level of mRNA expression of alkaline phosphatase and noncollagenous bone proteins in the samples extracted at the interface, only alkaline phosphatase mRNA expression was significantly higher at the interface between putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts. Although alkaline phosphatase and osteopontin were expressed strongly in putative epithelial rests of Malassez cells cultured alone and in human periodontal ligament fibroblasts cultured alone, the interaction has a strong influence on alkaline phosphatase expression. In fact, it can be easy that alkaline phosphatase expresses under various conditions, e.g., in mineralization medium which includes ascorbic acid, β-glycerophosphate and dexamethasone (66–68). There were no significant differences in osteopontin mRNA expression among the three cell populations. Osteopontin may not be directly associated with the interaction between putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts, although it can be associated with mineralization and cell adhesion in each cell (3,10–12,26,31). Tissue nonspecific alkaline phosphatase and osteopontin can be considered as differentiation markers because they are not exclusive to bone and cementum and they can be detected during the differentiation stages on proliferating precursor cells (3,10,52).

On the other hand, osteocalcin and bone sialoprotein have a very restricted tissue distribution and are almost exclusively produced by the hard tissue formation cells. The level of osteocalcin was increased at the interface between putative epithelial rests of Malassez cells and human periodontal ligament fibroblast cells, whereas osteocalcin mRNA was not detected in putative epithelial rests of Malassez

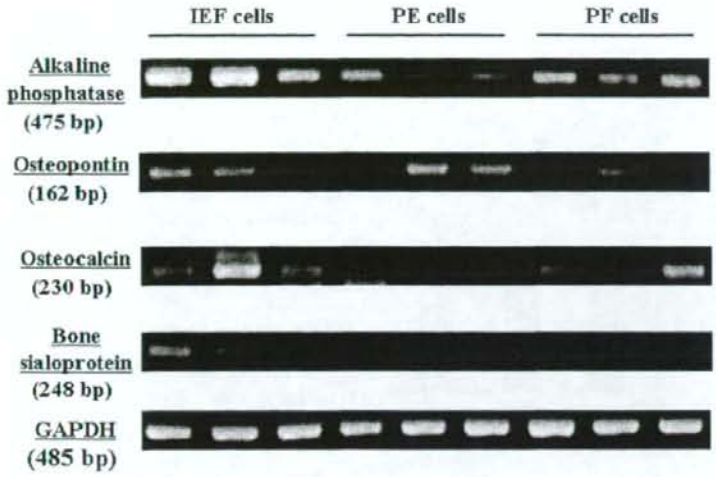

Fig. 5. Reverse transcription-polymerase chain reaction analysis of the mRNA expression of alkaline phosphatase, osteopontin, osteocalcin and bone sialoprotein in cultures of interface area cells, of putative epithelial rests of Malassez cells cultured alone and of putative human periodontal ligament fibroblasts cultured alone. The mRNAs were extracted from the samples including both putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts at the interface (interface area cells), putative epithelial rests of Malassez cells cultured alone, or human periodontal ligament fibroblasts cultured alone. Reverse transcription-polymerase chain reaction products were separated by electrophoresis on a 2% agarose gel and stained with ethidium bromide. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IEF, the cells including both putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts at the interface; PE, putative epithelial rests of Malassez cells cultured alone, PF, putative human periodontal ligament fibroblasts cultured alone.

cells cultured alone. Moreover, immunohistochemical and *in situ* hybridization data indicated that stronger expression and a wider distribution of osteocalcin were found in putative epithelial rests of Malassez cells at the interface compared with putative epithelial rests of Malassez cells cultured alone, but osteocalcin was not detectable in human periodontal ligament fibroblasts at the interface. The interface between putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts showed a stronger expression of bone sialoprotein mRNA compared with human periodontal ligament fibroblasts cultured alone, whereas immunohistochemical and *in situ* hybridization data indicated that bone sialoprotein was not detectable in putative epithelial rests of Malassez cells. When the RT-PCR findings of increased levels of osteocalcin and bone sialoprotein mRNA can reflect the immunohistochemical and *in situ* hybridization data, interaction between putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts facilitate osteocalcin expression in putative epithelial rests of Malassez cells and bone sialoprotein expression in human periodontal ligament fibroblasts.

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 (23). 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 (23–30). We also indicated that putative epithelial rests of Malassez cells have the ability to produce amelogenin, alkaline phosphatase and the noncollagenous proteins, osteopontin and osteocalcin, supporting their involvement in cementum formation. Bone sialoprotein was detectable in human periodontal ligament fibroblasts, but not in epithelial rests of Malassez cells. Previous reports indicated that bone sialoprotein is detectable in cementum and alveolar bone, but not in periodontal ligament *in vivo*, whereas alkaline phosphatase and osteopontin are detectable in normal periodontal ligament tissue (52,68). The origin of cementogenesis is still controversial. It appears that the extracellular matrix and the constructed cementum are formed by both epithelial rests of Malassez cells and human periodontal ligament fibroblasts, but it is difficult to identify the role of cementoblasts with certainty.

In contrast, immunohistochemical and *in situ* hybridization data indicated that osteocalcin was not detectable in human periodontal ligament fibroblasts in close proximity to epithelial rests of Malassez cells at the interface, while the RT-PCR showed that weak signs of osteocalcin mRNA could be detected in human periodontal ligament fibroblasts cultured alone. These findings suggested that the interaction between epithelial rests of Malassez cells and human periodontal ligament fibroblasts suppressed the production of osteocalcin in human periodontal ligament fibroblasts at the interface. Osteocalcin is one of the extracellular matrix proteins of cementum and bone that has been implicated to have a role in calcification. However, Ducy *et al.* reported that the finding of increased bone density in osteocalcin knockout mutant mice implicates osteocalcin as a potential inhibitor of bone formation (19). Aubin *et al.* showed that over a time course of osteoblast differentiation *in vitro*, the expression of type I collagen was found to be high and then to decrease; the level of alkaline phosphatase increased but decreased when mineralization was well progressed; osteopontin appeared before certain other matrix proteins, including bone sialoprotein and osteocalcin; bone sialoprotein was first detected in differentiated osteoblasts forming

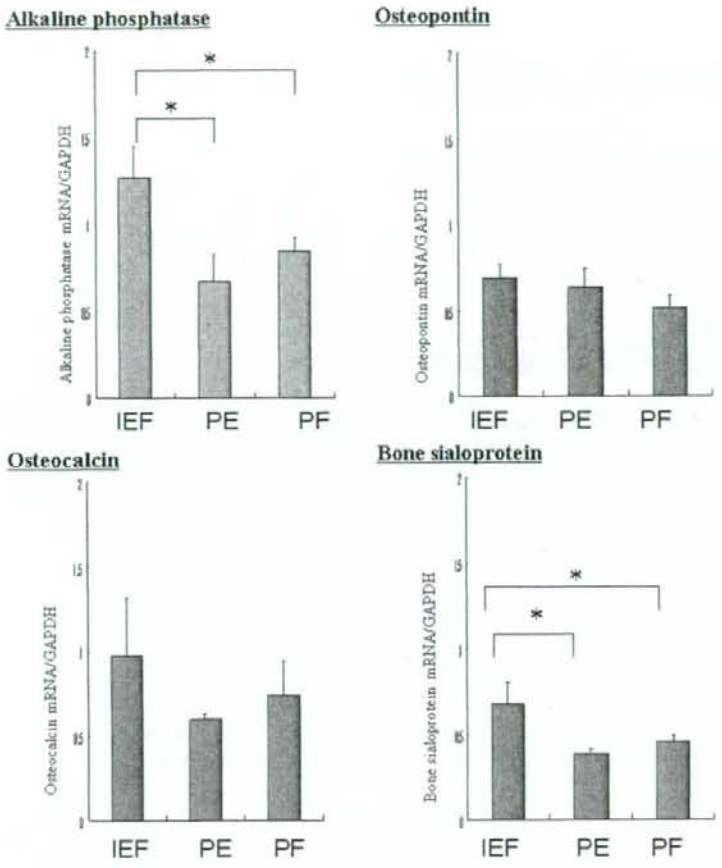

Fig. 6. Graphical representation of relative band intensities standardized by glyceraldehyde-3-phosphate dehydrogenase (mean ± standard deviation, $n = 3$; significant difference: *, $p < 0.05$). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IEF, interface area cells, comprising both putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts; PE, putative epithelial rests of Malassez cells cultured alone, PF, putative human periodontal ligament fibroblasts cultured alone.

bone; and osteocalcin appeared with mineralization (5). We showed, in the present study, that bone sialoprotein was detectable, but osteocalcin was not, in human periodontal ligament fibroblasts at the interface. Although human periodontal ligament fibroblasts have a potentially recognizable stage of differentiation, they may not divide and differentiate into the mature cells forming the hard tissues because of the invasion of the proliferating epithelial rests of Malassez cells at the interface.

Cell culture favors growth and selects for the most rapidly proliferating cells. Cell culture data should be cautiously extrapolated to the *in vitro* situation, particularly in providing evidence for cellular origins. However, this should not preclude the fact that valuable information can be obtained from *in vitro* studies, because the results from *in vitro* studies reflect various situations *in vivo*. Moreover, cells derived from periodontal ligament should produce the same proteins and mRNAs as those expressed in periodontal ligament tissues.

In conclusion, the data presented here indicated that the interaction between putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts modulates the expression of alkaline phosphatase, osteocalcin and bone sialoprotein in putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts *in vitro*. Considering the homeostasis of the periodontium, we suggest that epithelial–mesenchymal interactions are highly associated with mineralization in periodontal ligament and play a role in the maintenance of the periodontal ligament.

Acknowledgements

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Chairside evaluation of pH-lowering activity and lactic acid production of dental plaque: Correlation with caries experience and incidence in preschool children

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Objectives: Because caries activity may be related to dental plaque acidogenicity, a method was developed for chairside evaluation of pH-lowering activity and lactic acid production by dental plaque. Moreover, this study examined the association of these 2 factors with caries experience on oral examination and with caries activity by following caries incidence for 4 years in a group of preschool children. **Method and Materials:** A dental plaque sample (2.4 µL) was collected from sound buccal surfaces of maxillary primary second molars using a spoon excavator and placed onto the sensor area of a portable pH meter. Sucrose (30 µL, 228 mmol) was mixed with the plaque sample, and pH changes were monitored for 10 minutes. After pH determination, lactic acid concentration in the plaque-sucrose mixture was measured using a portable lactate meter. **Results:** Caries experience of subjects correlated with minimum pH (at 10 minutes after sucrose addition) ($r = -0.53$, $P < .001$) and lactic acid production ($r = 0.38$, $P < .001$). In addition, increments of both primary tooth caries (Δdft) and permanent tooth caries (ΔDFT) for 4 years correlated with minimum pH ($r = -0.47$, $P < .005$ and $r = -0.38$, $P < .05$, respectively). Setting cut-off values of pH and lactic acid concentration at 5.0 and 7.0 mmol/L for Δdft allows screening for caries-susceptible subjects (sensitivity = 0.950 and 0.800, specificity = 0.391 and 0.783, respectively). **Conclusion:** Plaque in caries-susceptible preschool children displays greater pH-lowering activity and lactic acid production. This method can be applied as a chairside screening test for caries activity and susceptibility for preschool children in dental clinics. *(Quintessence Int 2008;39:151–158)*

Key words: caries experience, caries incidence, chairside evaluation, dental plaque, lactic acid, pH

The acid productivity of dental plaque is considered one of the most significant caries risk factors, since dental caries is initiated through demineralization of the tooth surface by organic acid produced by dental plaque bacteria.[1] Previous studies have reported that after rinsing with sugar, dental plaque obtained from caries-active patients shows lower final pH than that from caries-inactive patients.[2,3] Lingström et al[4] reported that dental plaque covering enamel caries lesions displays higher pH-lowering activity than that from healthy tooth surfaces. All these findings suggest that assessment of pH-lowering activity of dental plaque is one of the first steps in evaluating dental caries risk.

Microorganisms in dental plaque produce various organic acids by sugar fermentation, including lactic, acetic, and formic acids.[5] Of these, lactic acid is the main product

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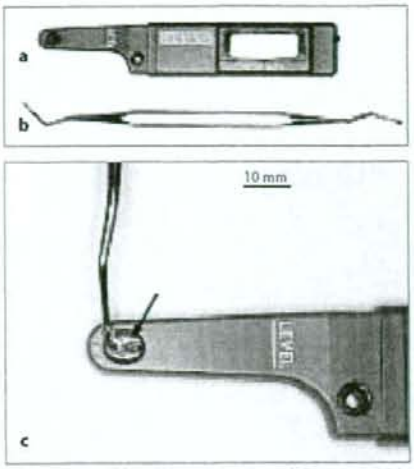


Fig 1 Portable pH meter (*a*, model pH BOY-P1, Sindengen Kogyo) and dental spoon excavator (*b* model No. 1, Yamaura Seisakusyo). A spoon-excavator-cup of dental plaque (2.4 µL) was immediately placed on the sensor of the pH meter (*c, arrow*) using a sterilized toothpick.

contributing to reductions in dental plaque pH at the initial phase of the Stephan curve.[6,7] In starved plaque from adults, concentration of lactate was higher in caries-positive subjects than in caries-free subjects, although the proportion of lactate among all organic anions was small.[2,3] After sucrose was added to dental plaque, only lactic acid increased among the organic acids,[8,9] and concentrations of lactic acid were higher in caries-positive subjects than in caries-free subjects.[3]

The association of caries activity with pH-lowering activity and lactic acid production of dental plaque as described above strongly suggests that evaluation of these factors in dental plaque is important for caries risk assessment. However, until now, no simple methods have been available for chairside evalustion of these 2 factors as dental plaque acidogenicity, and little information has been accumulated on relationships with dental caries activity in children. In this study, we first attempted to develop a method for chairside evaluation of pH-lowering activity and lactic acid production by dental plaque in preschool children. Second, we examined the association of dental plaque acidogenicity with caries experience by oral examination and with caries activity by following caries incidence for 4 years. Third, we attempted to determine cutoff points for pH-lowering activity and lactic acid production for screening subjects with high caries risk.

METHOD AND MATERIALS

Subjects and oral examination

Subjects comprised 51 preschool children (25 boys, 26 girls; mean age, 4 years 0 months; range, 2 years 10 months to 6 years 4 months) who visited Shimizu Pediatric Dental Clinic in Shizuoka City, Shizuoka Prefecture, Japan. No subjects were using orthodontic appliances, had been diagnosed with fluorosis, or were on medication. Subjects and their parents provided informed consent to participate in this study.

Oral examination was performed by one clinician to determine the number of decayed, filled teeth (dft) and the modified Oral Hygiene Index Simplified (OHI-S)[10] using a dental mirror and an explorer under sufficient light. In addition, dental radiographs were used if necessary. Teeth with caries lesions of C2 (dentin lesion), C3 (perforation to pulp), and C4 (existence of root only) were counted as "dt." However, no incidents of C4 were found in our subjects.

Measurement of dental plaque acidogenicity at first oral examination

After confirming that the subject had not consumed any food for at least 2 hours, dental plaque was sampled from sound buccal surfaces of maxillary primary second molars using a dental spoon excavator (model No. 1, Yamaura Seisakusyo). The volume of one cup of the spoon excavator is equal to 2.4 µL. A spoon-excavator-cup of dental plaque was immediately placed on the sensor area of a portable pH meter (model pH BOY-P1, Sindengen Kogyo) (Fig 1) using a sterilized toothpick and then mixed with 30 µL of 228 mmol/L sucrose solution. Changes in pH were monitored for 10 minutes at room temperature.

Table 1 Values of dft, minimum pH, lactic acid production, and OHI-S at the first oral examination, and dft, Δdft, DFT, ΔDFT, minimum pH, and OHI-S at the second oral examination

	Range	Mean ± SD
First oral examination (n = 51)		
dft*	0–16	4.1 ± 3.89
Minimum pH	4.2–5.9	4.8 ± 0.44
Lactic acid (mmol)	1.4–18.5	7.5 ± 3.8
OHI-S	0.5–2.0	1.1 ± 0.5
Second oral examination (n = 43)		
dft	0–16	5.8 ± 4.26
Δdft	0–10	2.4 ± 3.04
DFT	0–4	0.5 ± 1.16
ΔDFT	0–4	0.5 ± 1.16
Minimum pH	4.3–5.5	4.7 ± 0.25
OHI-S	0.0–1.0	0.6 ± 0.3

*Filled teeth at the first oral examination = 0.

The pH value at 10 minutes after sucrose addition was designated as minimum pH. As previously reported,[4] pH values reached a minimum within 10 minutes.

Promptly at 10 minutes, 3 μL of 500 mmol/L sodium phosphate buffer (pH 6.8) was added to the reaction mixture to neutralize the acidic mixture; concentrations of lactate were measured using a portable lactate meter (model Lactate Pro, Arcray). The lactate meter adopts an amperometric method using the lactate oxidase reaction with an optimal pH in the neutral area. Lactate concentration was designated as lactic acid production.

Measurement of dental plaque acidogenicity at second oral examination

At 4 years after the first oral examination, 43 of 51 children (19 boys, 24 girls; mean age, 8 years 0 months; range, 6 years 0 months to 10 years 4 months) underwent oral examination and measurement of pH-lowering activity of dental plaque as described for the first oral examination. Increments of primary tooth caries (Δdft) and permanent tooth caries (ΔDFT) were calculated.

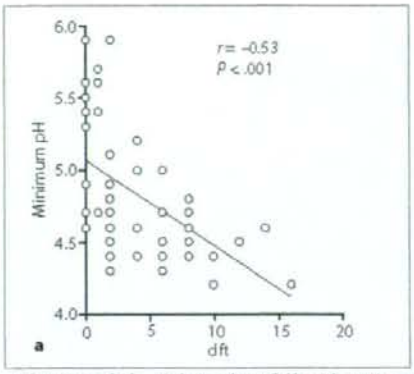
Statistical analysis

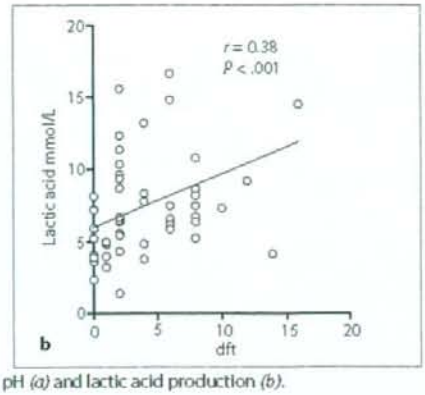
Correlations among minimum pH, lactic acid production, dft, DT, DFT, Δdft, and ΔDFT were evaluated by regression analysis and analysis of variance (ANOVA). To evaluate the predictability of minimum pH and lactic acid production for future caries incidence, receiver operating characteristic (ROC) curves were plotted from 2 × 2 tables showing distributions of subjects with and without caries increment at each cutoff value of minimum pH or lactic acid production. Sensitivity and specificity were also evaluated by χ^2 analysis.

RESULTS

All clinical data at the first and second oral examinations are summarized in Table 1. At the first oral examination, subjects with higher dft showed lower minimum pH ($r = -0.53$, $P < .001$; Fig 2a) and higher lactic acid production ($r = 0.38$, $P < .001$; Fig 2b). In addition, a significant correlation was identified between minimum pH and lactic acid production ($r = -0.74$, $P < .001$; Fig 3). No significant correlation was apparent between OHI-S and dft, or between OHI-S and minimum pH or lactic acid production. A similar correlation was observed between dft and minimum pH at the second oral examination ($r = -0.54$, $P < .001$).

Caries increment in primary teeth between the first and second oral examinations (Δdft) significantly correlated with minimum pH and lactic acid production at the first oral examination, but not with dft at the first oral





Figs 2a and 2b Relationship of dft with minimum pH *(a)* and lactic acid production *(b)*.

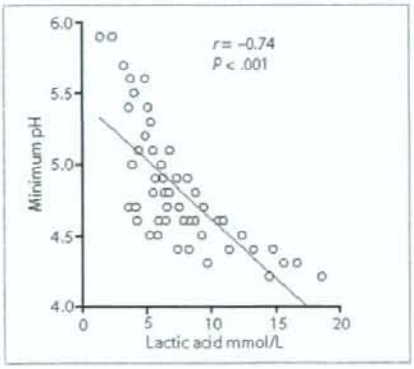


Fig 3 Relationship between lactic acid production and minimum pH.

Table 2 Correlation coefficient and probability of significance

First oral examination	Δdft	ΔDFT
Minimum pH	−0.47**	−0.38*
Lactic acid	0.66***	0.26
dft*	0.27	0.45**

*Filled teeth at the first oral examination = 0.
*$P < .05$
**$P < .005$
***$P < .001$

examination (Table 2). Caries increment in permanent teeth (ΔDFT) was significantly correlated with minimum pH and dft at the first oral examination, but not with lactic acid production at the first oral examination (see Table 2). No correlation was observed between caries increment and OHI-S (data not shown).

Figure 4a shows ROC curves for Δdft and minimum pH or lactic acid production. In general, the curve for lactic acid production was closer to the x = 0 axis and y = 1 axis, but the sensitivity of minimum pH was higher than that of lactic acid production when specificity was decreased. Setting the cutoff value for minimum pH at pH 5.0 screened subjects with new caries for 4 years with a sensitivity of 0.950 and a specificity of 0.391 ($P < .05$) (Fig 4b). Setting the cutoff value for lactic acid production at 7.0 mmol/L screened subjects with a sensitivity of 0.800 and a specificity of 0.783 ($P < .001$) (Fig 4c). Conversely, ROC curves for ΔDFT were close to the y = x line (Fig 4d), and no significant cutoff value was obtained for minimum pH or lactic acid production (Figs 4e and 4f).

DISCUSSION

Chairside measurement of minimum pH and lactic acid production of dental plaque

Caries-risk testing requires a method that is simple, rapid, and usable from chairside to identify subjects who will become diseased

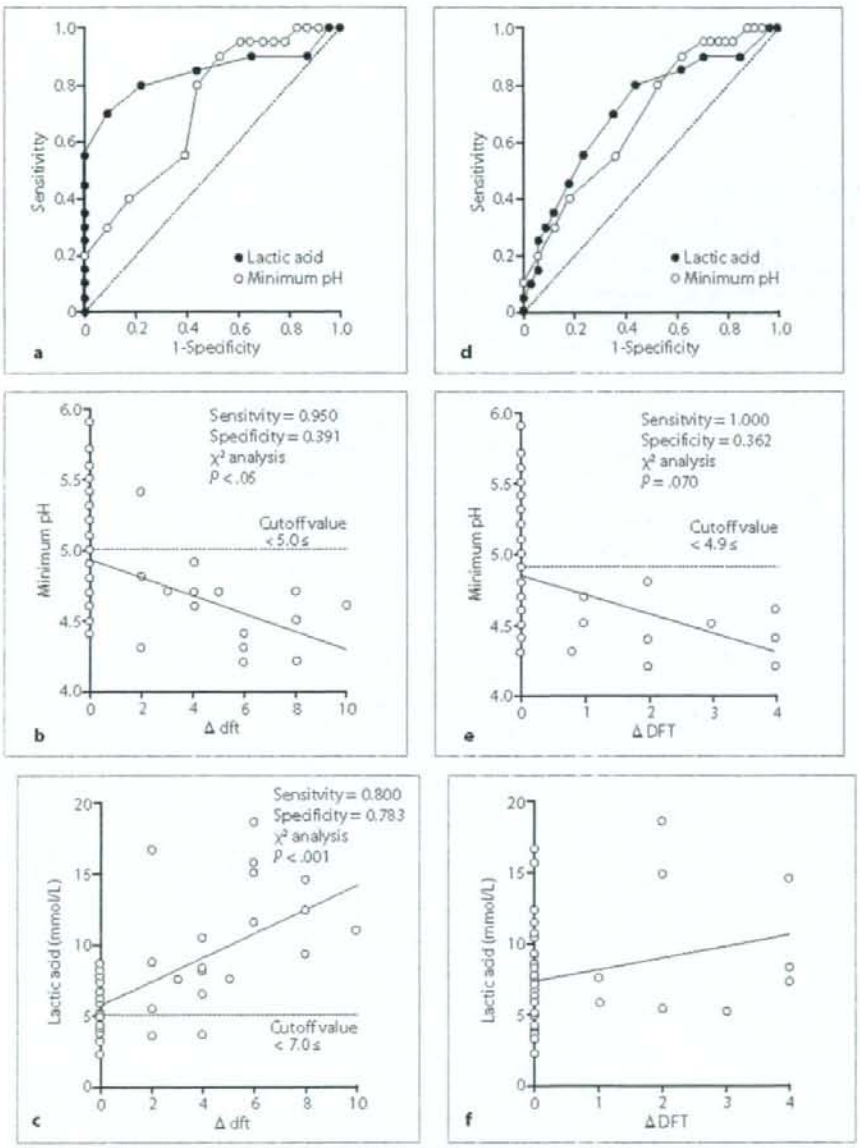


Fig 4 Relationship of Δdft and ΔDFT with minimum pH and lactic acid production at the first oral examination. ROC curves of Δdft versus minimum pH and lactic acid production *(a)* and ΔDFT versus minimum pH and lactic acid production *(d)*. Distribution maps showing Δdft versus minimum pH *(b)* and lactic acid production *(c)*, and ΔDFT versus minimum pH *(e)* and lactic acid production *(f)*. Cutoff values are indicated on each panel with corresponding sensitivity, specificity, and *P* value of χ^2 analysis.

and exclude subjects who will remain healthy.[11] In saliva tests evaluating caries risk, microbial counts of *mutans streptococci* and *lactobacilli* and biochemical properties (volume, pH, buffer capacity, etc) of saliva are usually evaluated. However, since dental caries is a phenomenon occurring at the interface between the tooth surface and dental plaque where salivation is limited, tests using saliva may not always clearly show caries status. In preschool children, the association between dft and salivary rate was not significant, while association between dft and salivary buffer capacity varies from study to study.[12-14] Although significant correlations have been observed between salivary *mutans streptococci* count and dental caries experience in preschool children,[15] this method is not rapid and efficient because of the cultivation procedure, which requires 24 to 48 hours.

Cariostat (Sankin), a caries-risk test evaluating acidogenicity (decreases in pH) of dental plaque by cultivation of dental plaque in sucrose-containing liquid culture medium, has been considered useful for screening high-risk infants[16] and predicting caries occurrence in children.[17,18] In addition, this test uses simple methods for sampling, analyzing, and managing.[19] However, the test requires 48 hours of cultivation to obtain results. Conversely, the method used in the present study was capable of measuring rapid sucrose-dependent falls in pH that occur naturally in the oral cavity. In addition, this method can be completed at chairside using a standardized amount of dental plaque.

Correlation of minimum pH and lactic acid production with caries experience

The results obtained by our method indicate lower minimum pH in preschool children with higher dft ($r = -0.53$, $P < .001$). Minimum pH after adding sucrose reportedly depends on the aging of dental plaque.[20-22] However, each subject in this study performed daily oral care (personal toothbrushing) with relatively low OHI-S (mean, 1.1 ± 0.5; range, 0.5 to 2.0), and no significant difference was apparent between OHI-S and minimum plaque pH. The difference in minimum plaque pH observed in our study may be attributable to individual variation rather than plaque age, eg, differences in acidogenic activity of dental plaque. Lingström et al[4] compared the acidogenicity of dental plaque from sound surfaces of adult subjects having low DMFS (decayed, missing, filled surfaces) with that of adult subjects having high DMFS, and reported that differences in minimum pH are relatively small among adults. This could be due to differences between adults and children, or differences in plaque samples. Subjects in the study by Lingström et al refrained from oral hygiene for 3 days to allow sufficient plaque formation, so that overaccumulation and maturity of dental plaque may have obscured individual variations in dental plaque acidogenicity. The correlation between DFT (= ΔDFT) and minimum pH at the second oral examination in the present study was not significant (see Table 2), also suggesting a difference between caries in primary and permanent teeth.

Minimum pH and lactic acid production as a caries activity indicator

High lactic acid production correlated with minimum plaque pH in this study (see Fig 3), indicating that pH reductions in dental plaque are mainly caused by lactic acid produced from sucrose under the conditions of this study. This is similar to the phenomena occurring in dental plaque after sucrose rinse.[23,24] A correlation between Δdft and both minimum pH and lactic acid production thus seems natural (see Table 2), suggesting the validity of minimum pH and lactic acid production as an indicator of caries activity for preschool children. Both correlation coefficient (see Table 2) and ROC curve (see Fig 4a) indicate that lactic acid production was more strongly related to Δdft, suggesting the superiority of lactic acid production as a caries activity indicator. When a cutoff value of 7.0 mmol/L was set, sensitivity and specificity of lactic acid production as caries predictors were 0.800 and 0.783, respectively, supporting the validity of this method for predicting caries incidence (see Fig 4c).