of Oral Surgery, Tohoku University Graduate School of Dentistry. Informed consent was obtained from the patients before extractions. After washing the teeth several times with alpha-minimum essential medium (α-MEM; Cosmo Bio, Tokyo, Japan) supplemented with 10% fetal bovine serum and antibiotics (60 $\mu g\ ml^{-1}$ kanamycin, 20 units ml^{-1} penicillin G, 10 $\mu g\ ml^{-1}$ fungison, Sigma, St Louis, MO, USA), PDL explants were carefully removed from the root with a scalpel. They were then plated in 35-mm culture dishes in α-MEM. The explants produced outgrowths that were primarily composed of fibroblasts. After 1 wk, α-MEM was replaced by a modified serum-free medium (3 : 1 (v/v) MCDB153 medium; Sigma) supplemented with 5 $\mu g\ ml^{-1}$ insulin (Sigma), 0.5 $\mu g\ ml^{-1}$ hydrocortisone (Sigma), 10 $\mu g\ ml^{-1}$ transferrin (Sigma), 14.1 $\mu g\ ml^{-1}$ phosphorylethanolamine (Sigma), 10 ng ml^{-1} epidermal growth factor (EGF; Sigma) and antibiotics (24–26): α-MEM) including 40 $\mu g\ ml^{-1}$ bovine pituitary extract (Kyokuto, Tokyo, Japan) (27–29). This resulted in outgrowths of epithelial cells next to fibroblast-like cells. 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 0.25% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA) solution 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)) (30).

Gingival tissues were removed from clinically healthy patients at the time of the third molar extraction. After mincing they were cultured as described above. Cultures of PDL epithelial cells only or PDL fibroblasts only were used as controls.

Growth curves

Epithelial cells from PDLs and gingival tissues (second subculture) were seeded at 2×10^4 cells cm^{-1} in 12-well culture plates (Falcon; Nippon Becton Dickinson, Tokyo, Japan). The number of cells per dish on successive days was determined using a hemocytometer after trypsinizing the cells from the dish.

Statistical analysis

Statistical analysis of data was performed using Student's *t*-test ($P < 0.05$).

Scanning electron microscopy

Outgrowths of both epithelial cells and fibroblasts from human PDL cultured on the dishes were fixed with 2.5% glutaraldehyde in PBS at 4°C for 1 h and processed according to the tannic acid technique of KATSUMOTO *et al.* (31). Specimens were dried in a critical point dryer (HCP-2; Hitachi, Tokyo, Japan), ion-sputtered with Au–Pd in a coating unit (Hitachi 01–101), and examined with a scanning electron microscope (Hitachi S-510).

Immunohistochemistry

Cells were fixed with 4% paraformaldehyde at room temperature for 10 min. Alternatively, cells cultured on plastic discs (Sumilon; Sumitomo Bakelite, Tokyo, Japan) were dehydrated using a graded series of ethanol treatments, and then embedded in paraffin. Serial sections of 10-μm thickness were cut on a microtome. Both the cells on the dish and the vertical sections were processed for immunohistochemical examination. To inhibit endogenous peroxidase, 3% hydrogen peroxide was added. The cells and the vertical sections were then incubated with 10% normal goat serum for 30 min to block non-specific binding. They were then treated at room temperature for 2 h with a primary antibody to monoclonal mouse antihuman cytokeratin AE1/AE3 (1 : 50) (Dako, Carpinteria, CA, USA) to detect epithelial cells. The cells and vertical sections were then incubated overnight at 4°C with primary antiserum to rabbit antiporcine 25 kDa amelogenin (0.1 $\mu g\ ml^{-1}$) (courtesy of Dr T. Uchida, Second Department of Oral Anatomy, Hiroshima University School of Dentistry, Japan), which recognizes both human and rat amelogenin (6, 32), and with primary antibodies to monoclonal mouse antihuman type IV collagen (1 : 500) (Sigma) or polyclonal rabbit antihuman laminin (1 : 50) (Rockland, Gilbertsville, PA, USA). After rinsing in PBS, the cells were incubated with biotinylated immunoglobulin at room temperature for 30 min and stained by the avidin-biotinylated peroxidase complex (ABC) method, using an ExtrAvidin peroxidase staining kit (Sigma) and an AEC (3-amino-9-ethylcarbazole) chromogen kit (Sigma).

Proliferating, DNA-synthesizing cells in co-cultures were identified by nuclear incorporation of 100 $\mu mol\ l^{-1}$ 5-bromo-2′-deoxy-uridine (BrdU; Boehringer Mannheim Biochemica, Germany) for 24 h. Labeled nuclei were detected with monoclonal mouse anti-BrdU antibodies for 30 min at 37°C. After washing three times in PBS, the cells were incubated with HRP-conjugated goat antimouse immunoglobulin (Chemicon International, Temecula, CA, USA) for 30 min at 37°C and stained with 3,3-diaminobenzidine (DAB) and H_2O_2. Mayer's hematoxylin solution was applied for counterstaining. For control staining, PBS was used instead of the primary antibody.

In situ hybridization

The oligonucleotide probes used for *in situ* hybridization were synthesized by Nihon Gene Research Laboratories, Sendai, Japan. The sequences were as follows:

Probe 1: 5′-TCC AGG GTA GCC CCT CTC TCC TTT TTC TCC CAA AGG TCC TGT GCC-3′ for type IV collagen α-1 mRNA (33);

Probe 2: 5′-GCC ACC AGG TGG TGT CGG CCT GGT TGT TGT AGT CGG TCA GGA AGG-3′ for laminin γ-1 mRNA (34).

A biotin label was added at the 3′ end.

A computer-assisted search (GenBank) using the above antisense sequences, as well as the corresponding sense sequences, revealed no significant homology with any known sequences, other than that of the type IV collagen α-1 chain and the laminin γ-1 chain, respectively.

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 PBS including RNase inhibitor and then digested with proteinase K (0.1 $\mu g\ ml^{-1}$) at room temperature. To inhibit endogenous peroxidase, 3% hydrogen peroxide was added. The specimens were hybridized with biotin-labeled probes in the hybridization solution in a humid incubation chamber overnight at 37°C. After washing

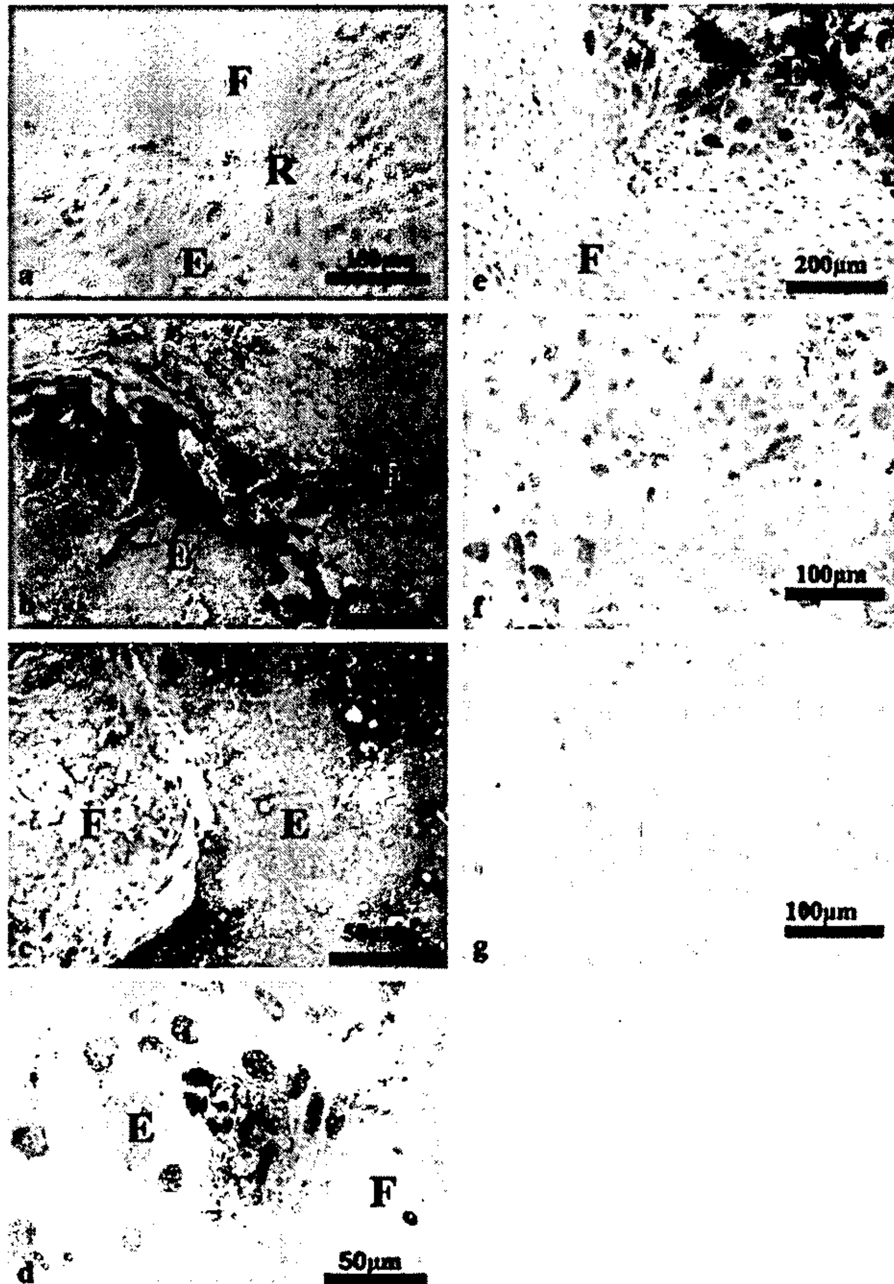

Fig. 1. (a) Phase-contrast photomicrograph of a primary coculture from human periodontal ligament. A sheet of epithelial cells (E) is covered by a ribbon (R) of fibroblasts (F). (b,c) Scanning electron micrographs of a primary cultures: (b) in the mixed multilayer, the epithelial cells (E) are seen to move under the fibroblasts (F); (c) epithelial cells (E) tightly attach to each other and push out the fibroblasts (F). (d) Photomicrograph showing assessment of proliferation by BrdU-labeling in epithelial cells (E) and fibroblasts (F) from human periodontal ligament. (e) Photomicrograph showing intense immunoreactivity for cytokeratin AE1/AE3 in epithelial cells (E) from human periodontal ligament. Immunostaining for cytokeratin in fibroblasts (F) is not seen. (f) Photomicrograph showing intense immunoreactivity for amelogenin in epithelial cells from human periodontal ligament; (g) immunostaining for amelogenin is not seen in epithelial cells from human gingival tissue.

in PBS, the specimens were reacted with blocking solution (5% BSA, 500 $\mu g\ ml^{-1}$ normal sheep IgG, 100 $\mu g\ ml^{-1}$ salmon testicular DNA, and 100 $\mu g\ ml^{-1}$ yeast tRNA in PBS) at room temperature for 15 min. They were then incubated with ExtrAvidin peroxidase solution (Sigma) at 37°C for 20 min, reacted with biotin-conjugated anti-avidin antibody (Sigma) in a humid chamber at 37°C for 30 min, and re-reacted with ExtrAvidin peroxidase solution. Following this, they were washed three times in PBS and the peroxidase sites were visualized using a solution containing

DAB and H_2O_2, with counterstaining using Mayer's hematoxylin solution.

Results

Characterization of cells in mixed cultures

Explant cultures from human PDL showed initial outgrowth of fibroblasts from the explant edges when maintained in α-MEM for 1 week. After replacing α-MEM with the modified serum-free medium, outgrowth of epithelial cells was induced in 50 out of 225 explant cultures (22.2%), and multilayers of both epithelial cells and fibroblasts were seen within 2–3 wk. All the co-cultures showed similar epithelial-mesenchymal interaction phenomena. When both epithelial cells and fibroblasts were cultured together in the modified serum-free medium, they were distinguishable from each other morphologically (Fig. 1a). Furthermore, scanning electron microscopy revealed that the epithelial colonies grew under fibroblasts (Fig. 1b), or attached adjacent to them (Fig. 1c).

Figure 2 shows typical growth curves of epithelial cells from PDLs and gingival tissues. The PDL epithelial cells had a lower growth rate than gingival epithelial cells under our culture conditions. In addition, PDL epithelial cells incorporated BrdU more extensively than PDL fibroblasts derived from the same PDL explant, indicating that PDL epithelial cells have a higher proliferation rate than PDL fibroblasts (Fig. 1d).

Immunohistochemistry

Human PDL epithelial cells in mixed cultures stained positive for broad-spectrum antibodies to cytokeratins (AE1/AE3), indicating their epithelial origin, while fibroblasts in the same culture did not show cytokeratin expression (Fig. 1e). Moreover, immunoreactivity for amelogenin was observed in human PDL epithelial cells, indicating their odontogenic epithelial origin, while human gingival epithelial cells did not show amelogenin expression (Fig. 1f,g).

In co-cultures (Fig. 3a) and vertical sections (Fig. 3c,d), the intensity of immunostaining for type IV collagen in human PDL fibroblasts immediately adjacent to human PDL epithelial cells was markedly high. Similarly, the intensity of immunostaining for laminin was increased at the interface (Fig. 3b). Immunostaining for both proteins showed little if any staining in areas further away from this interface. Control epithelial cells cultured alone showed only weak staining for type IV collagen (Fig. 3e) and laminin (data not shown). Control fibroblasts cultured alone showed little if any staining for type IV collagen (Fig. 3f) and laminin (data not shown).

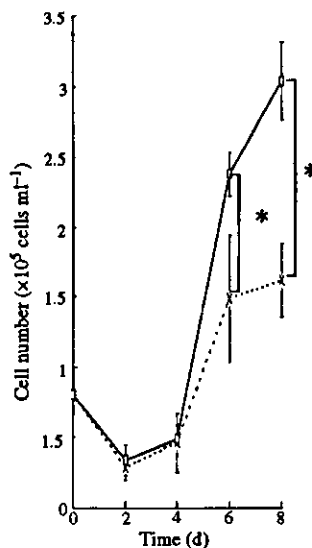

Fig. 2. Growth curves of epithelial cells from human periodontal ligament (dashed line with Xs) and gingival tissue (unbroken line with boxes). Values are the mean ± S.D. of five samples. $^*P < 0.05$.

In situ hybridization

To localize mRNA expression of type IV collagen α-1 chain and laminin γ-1 chain, the cells were hybridized *in situ* with biotin-labeled antisense oligo-DNA probes. The PDL fibroblasts immediately adjacent to PDL epithelial cells showed higher positive signals for both type IV collagen α-1 mRNA and laminin γ-1 mRNA (Fig. 4a,b). These results were consistent with the immunohistochemical observations (Fig. 3a,b). In the epithelial cells or the fibroblasts cultured alone, signals for type IV collagen α-1 mRNA and laminin γ-1 mRNA (data not shown) were weak compared with the co-culture.

Discussion

Both fibroblasts and epithelial cells were successfully cultured from human PDL tissue, using the explant technique in a modified serum-free medium. There were two steps in our co-culture system: in the first step, outgrowth of fibroblasts was induced from explants in α-MEM; and in the second step, outgrowth of epithelial cells was induced from the same explants, after replacing α-MEM with a modified serum-free medium. In this case, two different cell types were derived from the same tissue, but these cells did not mix, and instead formed fibroblasts as multilayers around epithelial cells. Keratin expression clearly distinguished epithelial cells from fibroblasts, since only the former cell type expressed this protein (Fig. 1e). In addition, amelogenin was expressed only in the cultured human PDL epithelial cells, and was not detectable in the human gingival epithelial cells (Fig. 1f,g). In a previous *in vitro* study, amelogenin synthesis has been reported in rat ameloblast-lineage cells (35), and these results suggest that our cultured human PDL epithelial cells were derived from the odontogenic epithelium, the epithelial cells of Malassez.

The morphological properties observed in the present study were similar to those found in an *in vitro* culture of monkey PDL fibroblasts and epithelial cells (22), and

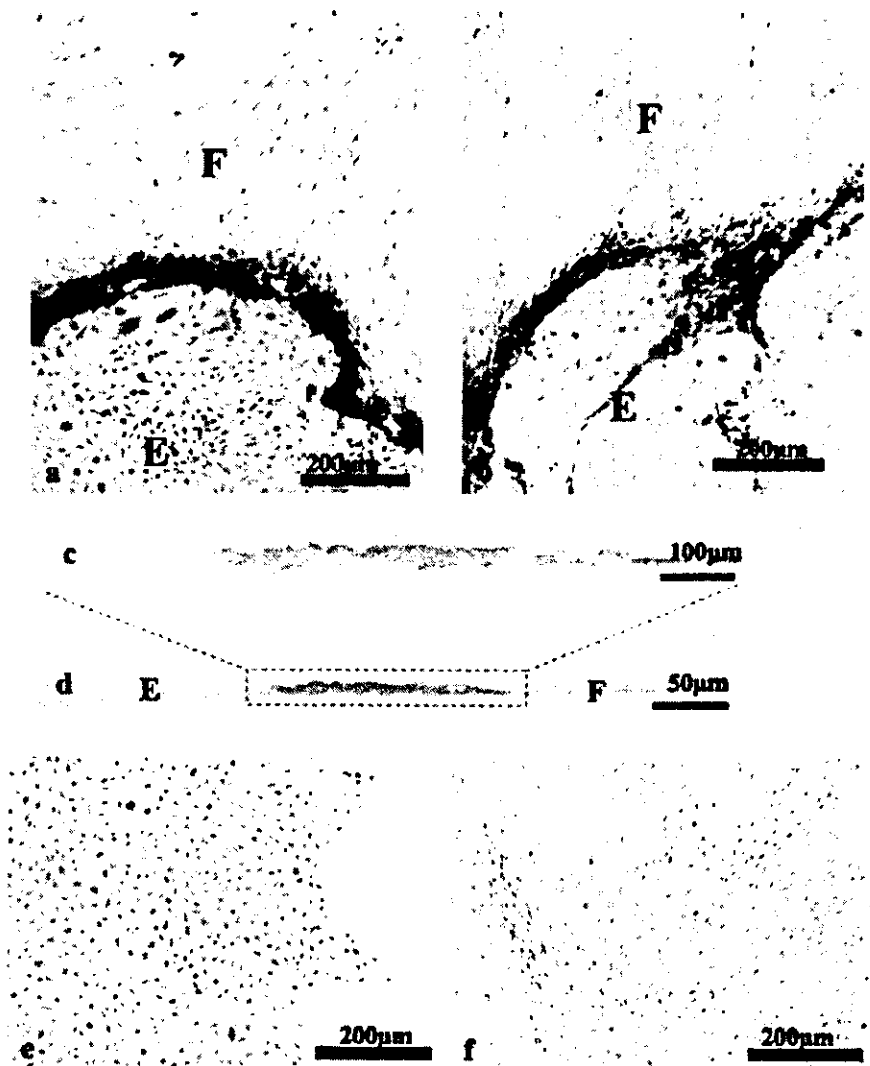

Fig. 3. (a,b) Photomicrographs showing intense immunoreactivity for type IV collagen (a) and laminin (b) between epithelial cells (E) and fibroblasts (F) from human periodontal ligament. (c,d) Photomicrographs showing intense immunoreactivity for type IV collagen at the interface between epithelial cells (E) and fibroblasts (F) from human periodontal ligament in vertical section. (e) Photomicrograph showing weak immunoreactivity for type IV collagen in control epithelial cells from human periodontal ligament. (f) Photomicrograph showing little immunoreactivity for type IV collagen in control fibroblasts from human periodontal ligament.

those of human PDL fibroblasts and mouse PDL epithelial cells (23). Ultrastructural changes of fibroblasts adjacent to the epithelial cells have also been reported for intracellular organelles, and have been related to protein synthesis, processing and transport (23). Additional features of interest in the ultrastructural studies were the presence of extracellular matrix between epithelial cells and fibroblasts under co-culture conditions (22, 23).

It is well established that extracellular matrix molecules regulate various physiological activities, such as cell growth, migration and differentiation (14–19). The basement membrane, a part of the extracellular matrix, separates the connective tissue from the epithelium and contains collagen (mainly type IV) and non-collagenous glycoproteins such as laminin. Under *in vitro* culture conditions, many cells secrete the components of the basement membrane (14, 15, 34, 36–41). In our study, enhanced expression and distribution of type IV collagen and laminin were found at the interface between epithelial cells and fibroblasts, while little staining for these proteins was apparent in areas further away from the interface of the mixed culture. These results suggest that interactions between epithelial cells and fibroblasts stimulate type IV collagen and laminin expression in epithelial cells *in vitro*. The modulation of the expression of extracellular molecules in fibroblasts by epithelial cells has been documented in previous cell culture studies (23), which showed that, under co-culture conditions, the synthesis of type I and III collagen and fibronectin was stimulated in PDL fibroblasts adjacent to PDL epithelial

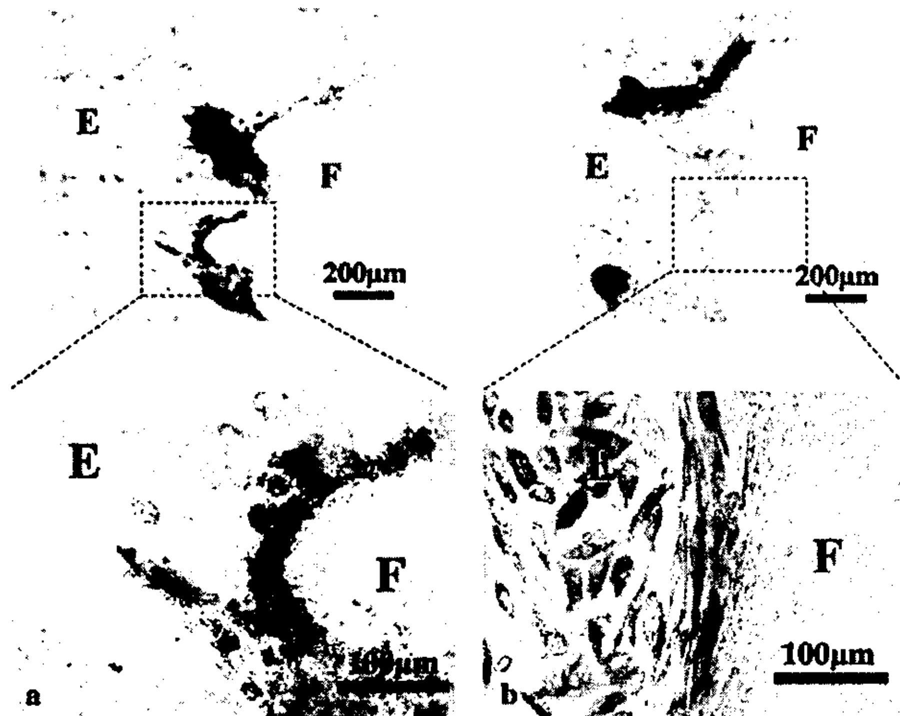

Fig. 4. Photomicrographs showing intense immunoreactivity for type IV collagen mRNA (a) and laminin mRNA (b) at the interface between epithelial cells (E) and fibroblasts (F) from human periodontal ligament.

cells. These observations are concordant with our observations, but the matrix proteins (type I and III collagen and fibronectin) detected are not components specific to the basement membrane. In another study in organotypic co-cultures of epithelial cells and fibroblasts derived from gingival tissue (41), type IV collagen and laminin were observed immunohistochemically underneath the basal epithelial cells. However, to our knowledge, our study is the first to show the synthesis of basement membrane constituents at the interface between epithelial cells and fibroblasts derived from PDL.

The effects of cell density and cell-cell contacts on protein syntheses appear very interesting. In our study, fibroblasts were clearly distinguishable from epithelial cells (Fig. 1a), and type IV collagen and laminin were strongly expressed only at the interface (Fig. 3a,b) where direct cell–cell contacts occurred, suggesting the involvement of such contacts in protein expression. During cell culture, an area of high cell density is sometimes produced, and in our study this area was observed in the culture of fibroblasts alone and epithelial cells alone (Fig. 3e,f), but significant protein expression was not observed in either of these cultures. Hence, these observations do not support the hypothesis that cell density itself affects protein expression. However, it is still unclear whether increased protein expression requires cell–cell contacts, synthesis of other proteins, or an appropriate cell density, and this remains to be elucidated.

The role of PDL epithelial cells in periodontal physiology is unclear. However, it is known that, when stimulated by endodontal infection, these cells can proliferate and give rise to periapical cyst formation (1, 42). It has been reported that epithelial cells derived from porcine rests of Malassez secrete prostaglandin, which possibly contributes to cyst growth (43). It has also been speculated that PDL epithelial cells may participate in periodontal diseases by proliferating during pocket formation and development of new junctional epithelium during postoperative gingival healing (2).

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