Pre-existing root cementum may promote cementoblast differentiation of human periodontal ligament cells

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Abstract

Objectives: To observe whether preserved healthy cementum could promote differentiation of human periodontal ligament cells to cementoblasts.

Materials and methods: Symmetrical root slices from each healthy premolar were distributed into either the control group (cementum removed) or test group (cementum preserved). After isolation and characterization, human periodontal ligament cells were inoculated onto root slices for 7 days co-culture. Two slices per group were studied for cell morphology by scanning electronic microscopy. Twenty-three slices were detected for expression of cementum attachment protein and cementum protein 23, two putative cementoblast markers, by real-time polymerase chain reaction. Twenty slices were transplanted into nude mice and analysed using histology and immunohistochemistry for osteopontin and bone sialoprotein expression after 8 weeks.

Results: Cells of the test group had smoother fibroblast morphology and higher cementum protein 23 and cementum attachment protein expression than those of the control group ($P < 0.01$). In the test group, 14 root slices revealed cementum-like matrix formation resting on old cementum; no splits were observed between newly formed matrix and old cementum. In the control group, 17 specimens had fibrous tissue formation along the root surface and varying width of splits could be seen between new fibrous tissue and dentine surface. Only three specimens demonstrated presence of

newly formed thin cementum-like matrix. Newly formed cementum-like matrix was positive for osteopontin and bone sialoprotein.

Conclusions: The results demonstrate that healthy root cementum may promote differentiation of human periodontal ligament cells towards cementoblasts.

Introduction

Cementum is a thin type of connective tissue that covers root surfaces, its major role being to serve as the site for anchoring principal collagen fibres. During the process of periodontitis, involved cementum experiences structural and biochemical changes (1). Based on presence of disease-mediating changes, complete removal of cementum has been advocated by most clinicians to re-acquire non-toxic root surfaces. Healthy root cementum would also be completely removed in the surgical experiment model of this. However, in contrast to this aggressive approach, more gentle treatment of the root surface, leaving most cementum in place while at the same time, removing and disturbing the attached bacterial biofilm, has been suggested by some researchers. At present, very little is known about the role of pre-existing cementum in periodontal regeneration; its organic matrix is composed primarily of collagens and is also a rich source of growth factors, which affect activities of various periodontal cell types (2). Pre-existing cementum on the root surface may serve as a beneficial microenvironment during periodontal wound healing.

Cementum regeneration is a crucial part of periodontal tissue reconstruction; however, outcomes of new therapeutic approaches, including guided tissue regeneration (GTR) and application of growth factors and enamel matrix proteins (EMPs) to root surfaces, remain debatable with regard to type of repair or regeneration

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(3). Findings from previous studies have indicated that in humans, cementum formed after treatment with GTR or EMPs is mainly cellular, and gaps are often present between newly formed cementum and underlying dentine, from which root cementum has been completely removed (4,5). These characteristics are considerably different from those of cementum formed during development. Although some researchers argue that artefacts are due to sample processing, frequent existence of such gaps is logical as surgical dentine surface is not suitable for cell growth and matrix formation. Some findings indicate that dentine components may induce release of inflammatory molecules and thus result in less periodontal tissue regeneration (6). Cementum covering dentine surfaces can prevent or reduce accumulation of inflammatory factors in the local environment (7). Some researchers have noticed that amounts of new cementum can lie directly on the top of old cementum, just apical to the periodontal defect (8). Some studies in dogs (9), show that new cementum formation is improved by preserving old cementum in GTR. Root cementum may modulate expression of growth and mineral-associated factors during periodontal regeneration in humans (10).

Cementum regeneration requires cementoblasts. Some studies indicate that the periodontal ligament (PDL) may be one source of cementoblast progenitors in adult humans. Such studies have demonstrated that a small proportion of cells cloned from human PDL had cementoblast-like characteristics (11,12). Stem cells in the PDL maybe a further source of cementoblasts (13). Differentiation of stem cells is most likely triggered by instructive and stimulatory signals provided by the local environment, which consists of the extracellular matrix (ECM) and growth factors, sequestered in the matrix (14). In periodontal wound healing, it is possible that pre-existing ECM of cementum may provide signals for stem cells in the PDL to differentiate into precursor cells and thus determine their differentiation into cementoblasts.

This study was designed to evaluate effects of different root surfaces on differentiation of PDL cells into cementoblasts. To evaluate this, we inoculated human periodontal ligament cells (hPDLCs) onto prepared cementum and dentine slices. Cell morphology, cementoblast-associated gene expression and tissue formation *in vivo* were analysed.

Materials and methods

Preparation of root slices

Healthy premolars were obtained with the written consent of human subjects, according to the Ethics Com-

mittee of Shandong University. Both crowns lower than 2 mm beneath the cemento-enamel junction, and the apical third part of the root were discarded. PDLs were removed completely by root scaling and gauze polishing. Roots were then cut in half longitudinally through the centre and all internal pulp was cleared. One symmetrical slice of each root was further scaled to remove root cementum and was assigned to the control group. The other symmetrical slice was assigned to the test group in which the cementum was preserved *in situ*. All slices were conditioned with 24% ethylene diamine tetraacetic acid (EDTA) gel, pH 6.5–7.2 (Biora AB, Malmo, Sweden) for 3 min, washed in copious quantities of distilled water and dipped into phosphate-buffered saline (PBS) supplemented with 10% penicillin and streptomycin, for 24 h. To confirm the procedure for the root slice preparation, four slices of each group were fixed in 4% paraformaldehyde, decalcified with 14% EDTA solution and processed for haematoxylin and eosin staining.

hPDLC isolation and stem-cell identification

Human periodontal ligament cells were isolated according to a modification of the method of Somerman *et al.* (15). Briefly, PDL tissues were taken from mid-roots of the extracted healthy premolars, cut into pieces and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 100 U/ml penicillin, streptomycin and 10% foetal calf serum (Gibco). Culture plates were kept at 37 °C in a humidified incubator (HERAccl, Newtown, CT, USA) in 95% air and 5% CO₂. When cells growing from explants reached confluence, they were detached using 0.25% trypsin (Gibco), and were subcultured into fresh flasks. Cells were observed using a reverse-phase microscope.

To make it clear whether hPDLCs present had stem cells amongst them, immunophenotyping for expression of human mesenchymal stem cell (hMSC)-specific cell surface marker was performed (16). Passage 2 hPDLCs were cultured on coverslips for 2 days. After fixation in 4% paraformaldehyde for 15 min, cells were rinsed in PBS and blocked with 10% normal goat serum for 45 min at room temperature. They were then incubated in diluted human STRO-1 antibody (R&D systems, Minneapolis, MN, USA) or mouse-IgG1 (as negative control) overnight at 4 °C. Following incubation with fluorescein-conjugated secondary antibody (R&D systems) at 37 °C in the dark for 60 min, cell nuclei were counterstained with DAPI, in the dark, for 3 min. Samples were mounted on slides then observed using a fluorescence microscope.

Inoculation of hPDLCs on root slices and scanning electron microscopy analysis

Root slices, approximately 7 mm × 5 mm × 1 mm in size, were placed in 48-well plates (Corning, NY, USA), one chip per well, with dentine or cementum surfaces orientated upwards. Second passage hPDLCs were harvested and suspended in DMEM at 1×10^6 /ml. Quantities of 20 µl of cell suspension were seeded sparsely on each root slice, followed by addition of 0.5 ml DMEM to each well. Cells and root slices were co-cultured under incubation, with media changed every 3 days. After 7 days incubation, two root slices from each group were fixed in 2% glutaraldehyde for 1 h and treated with 1% osmium tetroxide for 1 h. They were then dehydrated through a series of graded ethanol solutions, then coated with gold, and finally analysed by scanning electron microscopy (SEM, Fei Quanta 200; Eindhoven, Holland, FEI Co.).

Real-time reverse transcription polymerase chain reaction (real time RT-PCR)

After 7 days co-culture, hPDLCs growing on 23 root slices of each group were harvested and total RNA was extracted with Trizol reagent (Sangon, Shanghai, China). One microgram of RNA was reverse-transcribed using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, USA). One-tenth of the product was used for each 20 µl PCR reaction. PCR reactions were performed using LightCycler PCR Master Mix (Roche Diagnostics, Indianapolis, IN, USA). Specific primers for cementum attachment protein (CAP) and cementum protein 23 (CP-23) (Boya Corporation, Shanghai, China) were used. β-actin was included as internal control for amplification reaction. Sequence of primers is listed in Table 1. PCR products were resolved on a 2% agarose gel and visualized with ethidium bromide staining. Identity of PCR products was confirmed by sequencing.

For real-time PCR, LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics) was used. Master mix (18 µl) was placed in the glass capillaries and

2 µl of template DNA was added. Capillaries were placed in the rotor of the LightCycler (Roche Diagnostics Corp.). After denaturing at 95 °C for 10 min, temperature cycling was initiated. Each cycle consisted of denaturation at 95 °C for 10 s, hybridization at 59 °C for 5 s and elongation at 72 °C for 10 s. A total of 45 cycles were performed. Standard curves were analysed by LightCycler Software 4.0 (Roche Diagnostics) and relative amounts of transcripts were quantified after standardization with respect to amount of β-actin mRNA.

Transplantation of root slices

Ten BALB/C nude mice (4 weeks, 18–24 g; Beijing Laboratory Animal Research Centre) were used for this experiment, which was performed in accordance with the regulations and approval of the Institutional Animal Care and Use Committee, Shandong University. Animals were housed for 1 week to become acclimatized them to environment and diet of Shandong Laboratory Animal Research Centre.

On the eighth day of co-culture, 20 root slices in each group were rinsed in PBS and wrapped with sterilized expanded polytetrafluoroethylene (ePTFE) (Shanghai Plastics Research Institute, China). Mice were anaesthetized by intraperitoneal injection of 50 mg/kg chloramine. For each mouse an incision was made on the dorsal skin and a wrapped root slice was inserted beneath the skin; wounds were closed with sutures. Each mouse received transplants of four slices, two from each group. Penicillin was administered orally for one week to reduce risk of infection.

Histology and immunohistochemistry

All mice were sacrificed by neck dislocation 8 weeks post-surgery. Transplanted slices were excised, fixed in 4% paraformaldehyde for 1 day, then demineralized in 14% EDTA solution for approximately one and a half months, dehydrated in increasing concentrations of ethanol, cleared in xylene and embedded in paraffin wax. Longitudinal 5 µm serial sections were cut and processed for haematoxylin and eosin staining and for

Table 1. The primers for real-time RT-PCR

Gene	Primer sequence	<i>T_m</i> (°C)	Size (bp)	Accession number
CAP	5'-CCTGGCTCACCTTCTACGAC-3'	59.87	156	AY455942
	5'-CCTCAAGCAAGGC AAATGTC-3'	60.78		
CP-23	5'-GGCGATGCTCAACCTCTAAC-3'	59.84	158	NM_001048212
	5'-GATACCCACCTCTGCCTTG A-3'	60.07		
β-actin	5'-GATGAGATTGGCATGGCTTT-3'	59.36	160	NM_001101
	5'-CACCTTCACCGTCCAGTTT-3'	60.24		

immunohistochemistry. Primary antibodies were rabbit anti-human osteopontin (OPN, LF-123) and rabbit anti-human bone sialoprotein (BSP, LF-100), [both gifts from Dr. Larry W. Fisher (NIH, Bethesda, USA)], 1:100 dilution. Horseradish peroxidase conjugated goat-anti rabbit antibody (PV-6001; Golden Bridge International Inc., Beijing, China) was used as secondary antibody; its detection was performed by using diaminobenzidine (DAB) Liquid System (ZLI-9017, Golden Bridge International Inc.). Procedures were performed according to manufacturers' instruction. For negative control, primary antibody was substituted with PBS. All sections were counterstained with haematoxylin. Histological observation was performed by microscopy (OLYMPUS BX71, Tokyo, Japan).

Statistical analysis

Differences in CAP and CP-23 expression between control and test groups were evaluated by *t*-test and differences in tissue formation between control and test groups were evaluated by chi-square analysis. *P* value of < 0.05 was considered significant. All data were analysed using SPSS11.0 software.

Results

Isolation and stem-cell identification in hPDLCs

hPDLCs were successfully isolated from adult healthy PDL by the outgrowth method. Cells began to grow out from the PDL explants in 7–10 days. They then proliferated quickly and reached confluence by approximately 2 weeks (Fig. 1a). These cells were adherent to the tissue culture plates with spindle-shaped fibroblast morphology. Immunohistochemical staining revealed a selection of cells positive for STRO-1 antigen (Fig. 1b).

SEM observation of hPDLCs growing on root slices

Cell morphology of hPDLCs grown for 7 days on dentine and cementum surfaces was studied by SEM. Cells grew well and produced elongated cytoplasmic processes which connected to other cells, regardless of the different root surfaces. hPDLCs seeded and adherent to the dentine surface revealed extended shapes similar to those typical of fibroblasts grown on culture plates – long and spindle-shaped. These were flat and transparent, dentin tubules beneath cells remaining visible (Fig. 2a). On the other hand, cells cultured on the cementum surface had a little difference in morphology; they were a little larger, seemed less flattened and had

Figure 1. Human periodontal ligament cell (hPDLC) culture and the expression of mesenchymal stem cell-associated antigen in hPDLCs. (a) Microscopic observation of hPDLCs cultured by outgrowth method. Cells grew out from periodontal ligament explants (black arrows) and reached confluence at approximately 2 weeks. The cells had a spindle fibroblastic shape. (b) Immunohistochemical staining for STRO-1 in hPDLCs. Green fluorescence (red arrows) in cell plasma, sometimes manifesting the spindle shape of the cells, indicated that these cells were positive for Stro-1 antigen. The STRO-1 negative cells were observed by their blue stained nuclei (white arrows) (magnification 200 $\times$).

smoother fibroblastic shape compared to those growing on the dentine surface (Fig. 2b).

Gene expression of CAP and CP-23 in hPDLCs growing on root slices

Both CAP and CP-23 expressions were significantly higher on cementum surface cells than on those of the dentine surface ($P = 0.0035$ for CAP and $P = 6.50E-05$ for CP-23 respectively) (Fig. 3), suggesting that pre-

Figure 2. Human periodontal ligament cell (hPDL) colonization on the root surface after 7 day co-culture. (a) Dentin surface. The inoculated hPDLs grew well on the root surface with numerous processes connecting to other cells (black arrow). The cells were transparent and even the dentin tubule beneath them was clear to see (red arrow); (b) cementum surface. Note there was no dentin tubule visible. The seeded hPDLs were a bit larger and had a smoother shape than the cells grown on the dentin surface (black arrow) (SEM, magnification 400 $\times$).

served cementum had a positive effect on promoting differentiation of cultured hPDLs towards cementoblasts.

Tissue formation on transplanted root slices

On histological observation of root slice preparations, a thin layer of acellular cementum was observed along root surfaces of the test group (Fig. 4a), while dentine remained exposed in the control group (Fig. 4b).

Figure 3. Regulation of gene expression in human periodontal ligament cells after the cells were co-cultured with dentin and cementum root slice for 7 days. (a) Cementum attachment protein (CAP) expression in the control and test groups. (b) Cementum protein 23 (CP-23) expression in the both groups. In both (a) and (b), top panel showed the ethidium-bromide-stained agarose gel showing the RT-PCR fragments and the bottom panel showed the relative mRNA expression by analyzing the melting curves during real-time polymerase chain reaction procedure. Data were the mean of three independent experiments, and bars represented ($\pm$) SE. *Significant difference between the control and test groups.

Of root slice transplants, 20 specimens of each group were obtained. In the test group, 14 specimens revealed that there was a layer of cementum-like matrix formed on the surface of the old cementum (Fig. 4c). This newly formed cementum-like matrix (NFC) appeared less dense and varied in quantity and breadth amongst the different specimens, and even along the same root slice. Some specimens demonstrated that there were cells embedded in the NFC (Fig. 4e) and some displayed cells that were neither embedded nor aligned along the NFC (Fig. 5). Loose fibrous-like tissue always

Figure 4. Tissue formation by human periodontal ligament cells on cementum and dentin slices *in vivo*. (a) Cementum root slice showed a thin layer of acellular cementum (OC) along the root surface. (b) Healthy dentin root slice *in vitro* revealed the exposed dentin (black arrow). (c) A representative specimen showed there was newly formed cementum-like matrix (NFC) along the old cementum (OC) and there was a big gap between the NFC and fibrous-like tissue (black arrows). (d) Tissue formation on dentin slice. The representative dentin slice revealed fibrous tissue formation, and a wide gap was observed between the fibrous tissue and the root surface. The fibrous tissue was organized in a different way. The part closer to the ePTFE membrane (black arrows) was more loose than that towards the root surface (NF), but it was more like the fibrous tissue outside of the membrane (red arrows). (e) Magnification of panel in Fig. 5c. The newly formed tissue (NFC) was cementum-like but less dense and organized. A few cells were embedded and there was no bone marrow which distinguished cementum from bone. (f) Magnification of dentin slice. No cementum-like matrix formed on the dentin surface (red arrow). New fibrous-like tissue (NF) was split from the dentin surface and it was more dense than the adjacent fibrous tissue (black arrows). M, expanded polytetrafluoroethylene membrane (original magnification, a–c and f: 100 $\times$; d: 40 $\times$; e: 200 $\times$).

appeared between the NFC and the ePTFE membrane, with wide gaps between them (Fig. 4c). Fibrous tissue closer to the NFC was very thin, and was denser than fibrous tissue closer to the membrane (Fig. 5). However, of the fibrous tissue closer to the ePTFE membrane, both sides were much alike (data not shown). This suggests that transplanted hPDLs might also have formed thin fibrous-like tissue. Immunohistological results demonstrated that not all specimens expressed BSP and OPN. Staining for BSP was weak in newly formed matrix along the root surface and as greater the distance from old cementum was, stronger was the positive staining for BSP. Of the old cementum matrix and dentine, any staining for BSP was too faint to be clearly observed (Fig. 6a,b), but patterns of OPN staining were different from those of BSP. Staining of OPN was light-

to-strong in newly formed matrix on root surfaces, moderate in pre-existing cementum matrix, and no positive stain on dentine (Fig. 6d).

Of the 20 specimens in control groups, 17 had fibrous tissue formation and wide gaps were frequently observed between fibrous-like tissue and root surface (Fig. 4d). Fibrous tissue was organized in novel arrangement, that closer to the root surface was dense and parallel to it (NF in Fig. 4d,f) while parts closer to the ePTFE membrane (black arrows in Fig. 4d,f) was looser than NF; however, it resembled more the fibrous tissue external to the membrane (red arrow in Fig. 4d). From these histological characteristics it was learned that NF was formed by transplanted hPDLs, and fibrous tissue closer to the ePTFE membrane was derived from mouse cells. Only three specimens

Figure 5. Cementum root slice. Newly formed cementum-like tissue (NFC) lay directly on top of the old cementum (OC) with a lighter color distinction. This specimen showed neither cells embedded in nor aligned along the NFC. Fibrous tissue appeared between the NFC and expanded polytetrafluoroethylene membrane. This fibrous tissue was also composed of two parts. The part closer to the NFC (red arrows) was very thin and denser than the fibrous tissue closer to the membrane (black arrows) (original magnification, 200 $\times$).

revealed presence of a newly formed thin mineralization-like tissue on the root surface. More interestingly, in one specimen where cementum was not thoroughly removed, NFC was observed along the root surface where cementum was left. In comparison, areas where

dentine was exposed, only fibrous tissue was found (Fig. 7). New tissue formation in both groups is listed in Table 2. It shows that there were significantly more specimens with cementum-like matrix formation in the test group.

Discussion

Except for supporting the periodontal attachment apparatus, cementum also serves as a cover for dentine and protects it from resorption under pathological conditions. Even though exposed in the periodontal pocket, cementum is not greatly resorbed. Exposure of dentine *in vivo* allows osteoclasts to colonize the root surface and initiate the resorption process, which may evoke chemotactic and activator signals (17). From this point, cementum and dentine may have great differences in recruiting and regulating cellular events in the PDL. Clinically, both cementum and dentine act as mineralized connective tissue to which cells adhere, proliferate and differentiate, during periodontal wound healing. In our study, we performed root surface conditioning with EDTA for 3 min to mimic clinical treatment and to retain cementum and dentine ECM as intact as possible (18).

Our results showed that *in vitro*, both cementum and dentine surfaces were compatible for hPDLCs attachment and growth. After 7 d co-culture, morphology of cells grown on cementum surfaces changed somewhat compared to that of cells on dentine surfaces. This morphology change suggested that the process of cell differ-

Figure 6. Immunohistochemical stain for bone sialoprotein (BSP) and osteopontin (OPN) in cementum root slice. (a) Lower magnification image (original magnification, 100 $\times$) for BSP immunostaining. The staining for BSP was light in the newly formed cementum-like matrix (black arrow-heads) and no positive stain was detected in the pre-existing cementum and the dentin. (b) Magnification of panel in (a) showed dark brown stain (the black arrow) in newly formed cementum-like matrix (NFC) (400 $\times$). (c) Negative control of immunostaining. The primary antibody was substituted by PBS and no brown color was developed (200 $\times$). (d) Microscope observation of OPN immunostaining. This sample showed the staining for OPN was strong in the newly formed cementum-like matrix (black arrows showed the brown stain). The OPN stain for the pre-existing cementum was moderate (original magnification, 400 $\times$). OC, old cementum.

Figure 7. Distinct tissue formation on different root surfaces from the same root slice. One specimen showed new cementum-like tissue (white arrow head) formed on the surface of the pre-existing cementum (old cementum – OC), while no such tissue was present on the adjacent exposed dentin surface (the black arrow) (original magnification, 100 $\times$).

Table 2. Comparison of new tissue formation on the transplanted root slices

Groups	Total specimens	Number of specimens with cementum-like tissue formation	Number of specimens with fibrous-like tissue formation
Control	20	3	17
Test	20	14 ^a	6 ^b

^a and ^b: the test group compared with the control group, chi-square analysis ($P < 0.01$).

entiation could have happened. Cattaneo *et al.* have reported that cells in the presence of enamel matrix derivative (EMD) derive morphological changes that result in them being more similar to cementoblasts than to fibroblasts (19). EMD is an extracellular matrix that contains proteins similar to those from Herwig's epithelial root sheath (HERS). Cementum formed during such development is initiated by proteins secreted from HERS (20). It is possible that the cementum matrix may play a role similar to that of EMD and provide an environment favourable for hPDL cell differentiation.

Cementoblasts are the cells responsible for regeneration of cementum, but due to their similarity to osteoblasts, it is difficult to distinguish between the two cell populations. CAP has been characterized as a collagen-like protein, and serves as a marker for putative cementoblast progenitors of the adult human PDL (21). Cementum protein (CP) has been identified from conditioned medium of a human cementoblastoma-derived cell line. Among these, CP-23, found to be expressed by PDL cell subpopulations and cementoblasts, is now suggested as a tissue-specific gene product that can be a candidate to be a cementum-

specific marker (22). Human dental follicle cells, which are believed to contain precursor cells for cementoblasts, demonstrate CAP and CP-23 expression after EMD stimulation (23). We measured CAP and CP-23 mRNA expression in hPDL cells inoculated on root cementum and dentine surfaces. The results demonstrated higher mRNA expression of CP-23 and CAP in the cementum group, which suggests that pre-existing cementum might promote differentiation of hPDL cells towards cementoblasts.

To further verify our findings, we transplanted root slices, inoculated with hPDL cells, beneath the skin of the nude mice. Such mouse transplantation has been proven to be an effective model in which to study cell differentiation. ePTFE membrane can prevent murine cells infiltrating root slices during the first 3 weeks of transplant, and thus supply sufficient time for cells inoculated on the root surface, to grow and differentiate (24). Our *in vivo* results demonstrated that new matrix lay directly on the cementum surface and that this matrix was less dense and organized than the old cementum matrix. Some samples had low numbers of cells embedded in the matrix, but there was no bone marrow. From histological observation, this new matrix was cementum-like. We also performed immunohistological staining for BSP and OPN. These two proteins, which participate in initiating mineralization, are believed to play a major role in differentiation of cementoblast progenitor cells to cementoblasts (2). They are reported to be expressed in regenerated cementum, and also their expression is associated with new cementum attachment to dentine surfaces in a monkey model (25). OPN and BSP positive staining in our study indicated that newly formed matrix superficial to cementum was mineralization-related.

Except for matrix lying directly on top of old cementum, transplanted hPDL cells may also form a thin fibrous-like tissue. However, this fibrous-like tissue was much thinner than that formed by hPDL cells seeded on dentine slices. This suggests that cells transplanted on cementum surfaces formed cementum-like tissue and fibrous-like tissue, whereas hPDL cells seeded on dentine surfaces formed more fibrous-like tissues. This may be explained by heterogeneity of the hPDL cells.

The PDL contains various types of cells, including fibroblasts, endothelial cells, neural cells, osteoblasts and cementoblasts and mesenchymal stem cells; thus, cultured outgrown PDL cells were likely to be a heterogeneous population (26). Numbers of stem cells in a culture decrease with number of culture passages. Second passage cells used in this experiment contained certain quantities of stem cells, as shown by STRO-1 immunostaining, but numbers of stem cells were very much less than those of fibroblasts in PDL (26). When

hPDLs were inoculated onto root slices, cementum extracellular matrix was more suitable for stem cells or cementoblast progenitor cells to differentiate into cementoblasts, while fibroblasts amongst hPDLs formed thin fibrous tissue. However, dentine surfaces favour fibroblast growth, thus more fibrous tissues were formed in the control group. Based on our results, and previous findings [which have indicated that guanidine EDTA extracts of cementum can promote attachment of PDL cells, whereas guanidine EDTA extract of dentine does not (15)], we found that cementum matrix did affect cultured PDL cells in a different way from dentine matrix. We are not yet aware of the mechanism of the different tissue formation as our root slices were only surface-conditioned by EDTA, and proteins and growth factors were almost sequestered within the mineralized matrix. It is worthwhile exploring extracellular matrix differences between cementum and dentine after EDTA conditioning.

Complete removal of contaminated cementum has been advocated by some clinicians, but this aggressive debridement can damage and weaken the root. Our findings add evidence to a more gentle treatment proposal. Such gentler treatment, aimed at improving detoxification of the root surface while leaving most of the cementum, includes gentle scaling with hand instruments, conservative instrumentation with ultrasonic scalers or, on surfaces exposed during periodontal surgery, gentle conditioning with EDTA gels (18,27). Our study also sheds light on the application of cementum matrix derivative to exposed dentine surfaces (sometimes, dentine exposure is unavoidable, even with the gentler treatment method) to improve cementum regeneration. However, cementum exposed to periodontal pockets behaves differently from when it is healthy. Our ensuing studies will focus on gently treated diseased cementum, and explore its role in regulation of hPDL bioactivity.

Conclusions

In this study, we preserved cementum on the root surfaces by gentle scaling and sterilization. EDTA-conditioned cementum can enhance cementoblast-associated gene expression and cementum-like matrix formation on top of old cementum. Thus, pre-existing cementum may promote differentiation of inoculated hPDLs towards cementoblasts. We strongly suggest that healthy root cementum should be protected during periodontal surgery procedures.

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