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Periodontal regeneration following transplantation of proliferating tissue derived from periodontal ligament into class III furcation defects in dogs

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ABSTRACT
The aim of this study was to evaluate the healing of class III furcation defects following transplantation of proliferating tissue derived from periodontal ligament (pPDL). Two weeks after removing alveolar bone, pPDL was excised. Class III furcation defects were created in the mandibular premolars. pPDL was transplanted into the furcation defects in the experimental group, while no treatment was performed on the furcation defects in the controls. Two, four and eight weeks after surgery, histologic examination, quantitative RT-PCR, and immunohistochemistry were carried out. bFGF and VEGF mRNA showed a significant increase in pPDL. In the pPDL treatment group, new cementum regenerated around almost the entire circumference of the furcation, with new bone filling most of the defect, while the control group presented epithelial downgrowth and defects filled with connective tissue. These results provide histological evidence that pPDL plays an important role in wound healing by promoting periodontal regeneration in class III furcation defects.

Periodontal regeneration aims at the restitution of supporting periodontal tissues lost due to periodontal diseases (2). The effectiveness of various growth factors has been demonstrated by studies using animals on periodontal regeneration (3, 14). Guided tissue regeneration (GTR) therapy has been reported to achieve repopulation of periodontal ligament (PDL) fibroblasts and to promote periodontal regeneration in animals and humans (7, 9). Sugimoto et al. (26) investigated whether proliferating tissue derived from periodontal ligament (pPDL) in periodontal osseous defects could form new periodontal ligament. Immunohistochemical studies of pPDL revealed the presence of a number of matrix macromolecules such as collagen types I, III, and IV, vitronectin, fibronectin, bone sialoprotein, and osteonectin (1, 13). This discovery was consistent with the findings reported by Ivanovski et al. (12). They reported that mRNA expression of alkaline phosphatase, osteonectin, bone sialoprotein, and osteopontin increased in pPDL (12). The results of those studies suggest that periodontal regeneration is achieved through pPDL because of its abundant matrix components specific to distinct periodontal components. pPDL applied to the surface of dentin block implants has recently been investigated by in vivo study (26). Over an 8-week observation period, the implanted root bearing pPDL formed new PDL on the surface. Therefore, the authors concluded that pPDL promoted new PDL formation around the root (26). Lang et al. (16) reported similar results in dogs. These studies indicated that various cells migrated from pPDL which were basically capable of synthesizing periodontal tissue after transplantation.

The classification of the furcation defect is based on the amount of periodontal tissue; class I: horizontal loss of periodontal support not exceeding 1/3 of the width of the tooth, class II: horizontal loss of
periodontal support exceeding 1/3 of the width of the tooth, and class III: horizontal “through and through” destruction of the periodontal tissues in the furcation area. Various surgical procedures have been developed for healing class II or III furcation defects (4, 6), and various bone grafts and alloplastic materials have been transplanted (5, 24, 29); however, ankylosis, resorption, and long epithelial attachment are observed in alloplastic material-applied cases (8). It has been demonstrated that GTR is capable of successfully closing class III furcation defects in a dog model (11, 17, 18); however, the regeneration of class III furcation defects is often incomplete, even when GTR is employed, suggesting that this treatment is not effective for large, class III furcation defects (15, 21, 22), as it depends on natural healing ability. Since pPDL is basically capable of synthesizing periodontal tissue after transplantation, this treatment may be applicable to large periodontal tissue defects. Therefore, application of pPDL to class III furcation defects offers an attractive proposition.

The objective of this study was to investigate the effect of pPDL on periodontal tissue formed after transplantation in class III furcation defects in dogs.

MATERIALS AND METHODS

Fifteen healthy mongrel dogs were used for this study. The animals were placed under general anesthesia with ketamine hydrochloride (KETALAR®50; SANKYO, Tokyo, Japan) at a dosage of 10 mg/kg. In order to reduce haemorrhage in surgical areas, a local infiltration anesthesia (2% Xylocain®, 1:80,000 epinephrine; Astellas, Tokyo, Japan) was also used. The second mandibular premolars (P2) were extracted for pPDL as described below. All experiments were performed according to the Guidelines for the Treatment of Experimental Animals at Tokyo Dental College.

Preparation of pPDL. Three months after the extractions of P2, at 2 weeks before the experiment commenced (day 0), periodontal osseous defects were created around mandibular P1 to induce tissue growth under the membrane (hereafter referred to as pPDL). This method for induction of pPDL has been described previously by Sugimoto et al. (26). The root surface was then thoroughly scaled and planed. The defect was next covered with an e-PTFE membrane (GTAM Oval-9; WL Gore & Associates, Flagstaff, AZ, USA). The flaps were then replaced and sutured. Two weeks after surgery at experiment day 0, a re-entry surgical procedure was performed on the mandibular P1 to remove the membranes and obtain the pPDL, which was then excised. In this study, pPDL was used for transplantation. The harvested tissue was kept moist in sterile saline until used. Part of the pPDL was also used to examine mRNA expression of basic fibroblast growth factor (bFGF) and vascular endothelial cell growth factor (VEGF) using a LightCycler™ (F. Hoffmann-La Roche Ltd, Basel, Switzerland).

Surgical procedure. Five weeks before experiment day 0, class III furcation defects were created surgically at the third and fourth mandibular premolars using bone chisels and slow-rotation diamond burs. The alveolar bone of each premolar (P3, P4) was also removed, creating a “horizontal” pattern of bone loss (Fig. 1). The furcation defects were approximately 4 mm wide and 4 mm high and were filled with impression material (Exafine®, G.C. Corp. Tokyo, Japan). The flaps were repositioned and stabilized with silk sutures. The impression material was removed from the defects 2 weeks before experiment day 0. At experiment day 0, after elevation of the mucoperiosteal flaps of the class III furcation defects, granulation tissue was removed and the root surface was thoroughly debrided using curets. A notch was placed on either side of the root at the level of the reduced bone crest. During this time, the pPDL was retrieved as described above. On one side of the class III furcation defects for the mandibular premolars (experimental groups), pPDL was transplanted into the horizontal furcation defects. The contralateral premolars (control group) received the same treatment, but pPDL was not applied. Mucoperiosteal flaps were raised and sutured in a coronal position.
Periodontal regeneration of furcation defect by pPDL

**Histological processing.** The animals were euthanized with an intravenous overdose of sodium pentobarbital at two, four, and eight weeks following the procedure described above. The jaw of each animal was then removed, and specimens containing the experimental areas were placed in 20% buffered formalin. Specimens were decalcified with 10% ethylenediaminetetra acetic acid (EDTA; Wako, Tokyo, Japan) for 5 months and then dehydrated in ethanol, embedded in paraffin, and serially sectioned (to 4 μm thickness) in the mesio-distal orientation. The sections were then stained with hematoxylin-eosin. Three sections, 100 μm apart, representing the central area of each furcation, were selected from each tooth for linear measurements. In each section, the following linear distances were assessed: (1) the distance from the line marking the apical border between the notches on the mesial and distal roots to the fornix of the furcation, i.e., the height of the defect; (2) the distance from the line marking the apical border of the notches on the mesial and distal roots to the most coronal level of the newly formed alveolar bone, i.e., the height of the newly formed bone; (3) the circumference of the defect between the apical borders demarked by the notches on the mesial and distal roots; and (4) the total distance along the apical borders from the notches to the coronal level of newly formed cementum on the mesial and distal roots, i.e., the amount of new cementum. All distances were measured in millimeters (mm). The newly formed bone was also expressed as a percentage (%) of the height of the defect and the amount of new cementum as a percentage of the total circumference of the defect. Measurements were made using the following image analysis system: a light microscope (Olympus BX51 Microscope, Olympus Optical Co., Tokyo, Japan) with a × 4 objective, equipped with a digital camera (HC-500, FUJI FILM, Tokyo, Japan) coupled to a computer (DELL, Precision Work Section 220) with software used for image processing (Image Pro Plus v 3.0 Media Cybernetics, Silver Spring, MD, USA). Analysis of variance and the multiple comparison Scheffé test were used to analyze the data.

**Preparation of samples for quantitative RT-PCR using LightCycler™.** A re-entry surgical procedure was performed to obtain biopsies of pPDL (the proliferating tissue group) during the 2-week period after the surgery described above. Samples of the connective tissues of the gingiva (the connective tissue group) were separated from other gingival tissues. Total RNA was extracted from these samples by the acid guanidine thiocyanate/phenol-chloroform method. The samples were excised, then homogenized and solubilized in TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and chloroform. Supernatants were obtained by centrifugation at 13 200 rpm for 20 min at 4°C, added to isopropanol, stored for over 1 h at −80°C, and finally centrifuged at 13 200 rpm for 20 min at 4°C. The precipitates were obtained by decantation, and washed with 70% ethanol. The RNA pellets were dissolved in RNase-free water, and kept at −80°C until used. Total RNA was measured by absorbance in an UVmini-1240 (Shimadzu Corporation, Kyoto, Japan). Oligo dT primer: 1 μL, dNTP: 2 μL, RNase inhibitor: 1 μL, reverse transcriptase: 1 μL, 10× buffer: 2 μL, MgCl₂: 4 μL were added to a total of 1 μg of RNA, and the total volume was adjusted to 20 μL with RNase-free water. The mixture was then reverse transcripted (42°C for 15 min, 99°C for 5 min) to synthesize cDNA. PCR was then carried out using primers for bFGF, VEGF, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 1). To measure mRNA levels, quantitative PCR assays were conducted with a LightCycler™ using double-stranded DNA dye SYBR Green I (Roche Diagnostics, Manheim, Germany), and quantification was performed by comparing the levels obtained to standardized samples. The PCR conditions used in the LightCycler™ were: 45 cycles bFGF (95°C for 10 sec, 62°C for 10 sec, 72°C for 6 sec); 45 cycles VEGF (95°C for 10 sec, 57°C for 5 sec and 72°C for 8 sec); 45 cy-

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<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Product size</th>
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<tr>
<td>bFGF</td>
<td>5'-CAATACCTTACCAGGTCAAGG-3'</td>
<td>103bp</td>
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<tr>
<td></td>
<td>5'-TATAGCTTTCCTGCCCAAGG-3'</td>
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<tr>
<td>VEGF</td>
<td>5'-AGGAGTTCAACATCACCAT-3'</td>
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<td>GAPDH</td>
<td>5'-TGCGAAGGATGCGCCTGAC-3'</td>
<td>168bp</td>
</tr>
<tr>
<td></td>
<td>5'-CGGCCAGGGTCAATCCCAACT-3'</td>
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**Table 1 Primer sequences and product sizes**


Immunohistochemistry for PCNA. We carried out the following immunohistochemical analysis with mouse anti-proliferating cell nuclear antigen (PCNA) primary antibody. Paraffin sections (approximately 4 μm thick) were cut, and immunohistochemical staining of PCNA was performed using an immunoperoxidase staining kit (Histofine SAB-PO (M) kits; NICHIREI, Tokyo, Japan). The sections were incubated with mouse anti-PCNA primary antibody (PC-10; DAKO Corporation Carpinteria, CA, USA) at a dilution of 1:100. Next, each section was incubated with biotinylated secondary antibody and streptavidin peroxidase reagents. The presence of peroxidase-complexes was visualized by 3-3‘diaminobenzidine tetrahydrochloride (0.1 mg/mL) solution with 0.65% H₂O₂. Sections were counterstained with Mayer’s hematoxylin. Magnification was set at ×400. A field of connective tissue from immediately beneath the coronal to the apical area of the furcation defect was selected in each section, and a 0.12 mm² (0.2 mm × 0.6 mm) area was submitted to quantitative analysis. The number of PCNA-positive cells in the furcation area was calculated. An analysis of variance and the multiple comparison Scheffé test were used to analyze the data.

RESULTS

Two weeks after surgery
In experimental group, the furcation lesion was occupied by slightly inflamed and newly formed connective tissue (Fig. 2A). The slightly inflamed connective tissues that occupied the coronal half of the lesion were composed of many fibroblasts and collagen fibers, a small number of inflammatory cells, and primary lymphocytes adjacent to small blood vessels (Fig. 2B). The newly formed connective tissue (asterisk in Fig. 2A), which extended into the interradicular area, was distinguished from the slightly inflamed tissue by the presence of many blood vessels, abundant collagen fibers, and numerous well-polarized fibroblasts (Fig. 2B). In control group (data not shown), the most coronal portion of the lesion contained bacterial plaques on the root surface. The furcation defect was occupied by inflammatory connective tissue covered by gingival epithelium. The middle portion of the connective tissue contained more inflammatory cells, and the apical portion was composed of new fibrous connective tissue.

Four weeks after surgery
In experimental group, the furcation lesion was completely healed, filling with new fibrous connective tissue and bone (Fig. 2C). The coronal half was filled with connective tissue, whereas the apical portion had formed new bone on the alveolar bone. The fornix root surface of the furcation was covered with thick collagen fibers. Active bone formation was observed in the interradicular area (Fig. 2D). In control group (data not shown), the coronal half of the lesion was occupied by the gingival epithelium and slightly inflamed connective tissue beneath the gingival epithelium. The connective tissue was composed of fibroblasts and a small number of inflammatory cells. The apical portion was repaired and formed a small amount of new bone.

Eight weeks after surgery
In experimental group, the lesion had almost regenerated, with new cementum along the root surface, new bone and new PDL (Fig. 2E). The root surface of the furcation area was almost completely covered with new cementum, and Sharpey’s fibers inserting into the new cementum were observed (Fig. 2F); however, complete alveolar bone reconstruction was not achieved. Bone ankylosis and root resorption were not observed on the root surface. In control group, all furcations were open, showing inflamed connective tissue covered by gingival epithelium in the lower portion of the defect, and new cementum with inserted collagen fibers and new bone were limited to the level of the notch (Fig. 2G).

Immunohistochemistry for PCNA
The staining results for PCNA at week 4 after surgery in the newly formed connective tissue are shown in Fig. 3A. The experimental group showed more PCNA-positive fibroblast-like cells than the control group (Fig. 3B). Results indicating the number of PCNA-positive cells in the furcation area are summarized in Table 2. PCNA-positive cells in the experimental group were significantly greater than
in the control group (36.3 ± 2.5 vs 10.9 ± 2.1: p < 0.01).

**Histomorphometric Measurement**

Efficacy of treatment in the experimental group was evaluated by histomorphometric analysis of furcation defect regeneration at 8 weeks after surgery. The results are summarized in Table 3. The experimental group showed accelerated periodontal regeneration with new cementum by 98.1 ± 2.3%, and new bone by 84.8 ± 3.9%. In contrast, new cementum and new bone in the control group were 22.3 ± 1.6% and 12.2 ± 1.3%, respectively. This demonstrated that the amounts of new cementum and bone in the experimental group were significantly higher (p < 0.01) than in the control group.

**mRNA expression analyses of proliferating tissues**

Expression of bFGF and VEGF mRNA was investigated at week 2 after surgery with RT-PCR using a LightCycler™. This revealed that bFGF mRNA expression was detectable in the majority of samples from the pPDL group. It also showed that mRNA expression of bFGF and VEGF was higher in the pPDL group than in the connective tissue group (Fig. 4). The bFGF mRNA levels were significantly higher in the pPDL group than in the connective tissue group (p < 0.01) (Fig. 4B). VEGF mRNA expression was detected at significantly higher levels in the pPDL group than in the connective tissue group (p < 0.01) (Fig. 4C).
sequence of events accompanied by the formation of new cementum, PDL, and bone.

We found that local application of pPDL to class III furcation defects significantly increased periodontal regeneration without ankylosis or epithelial downgrowth. We also confirmed that local application of pPDL to class III furcation defects accelerated

**DISCUSSION**

We investigated whether local application of pPDL to class III furcation defects could effectively induce periodontal regeneration. It was demonstrated that pPDL treatment successfully healed class III furcation defects, and that the healing process involved a sequence of events accompanied by the formation of new cementum, PDL, and bone.

We found that local application of pPDL to class III furcation defects significantly increased periodontal regeneration without ankylosis or epithelial downgrowth. We also confirmed that local application of pPDL to class III furcation defects accelerat-
Periodontal regeneration of furcation defect by pPDL

Periodontal regeneration in the region to which pPDL was applied was accompanied by new cementum in 98.1 ± 2.3% of the circumference of the defect, and new bone in 84.8 ± 3.9% of the height of the defect. In the control region, in contrast, regeneration was accompanied by new cementum in 22.3 ± 1.6% of the circumference of the defect and new bone in 12.2 ± 1.3% of the height of the defect. These findings show that the wound healing process induced by pPDL treatment is important for new cementum and new bone formation. At 2 weeks after regeneration therapy using pPDL, the furcation defect was composed of fibrous connective tissue. At 4 weeks, the entire furcation was filled with fibrous connective tissue and this tissue at 8 weeks was replaced by new bone, PDL, and cementum. No inflammation or edema was noted in any region in the 2 weeks group of the pPDL treatment group. The furcation area was not exposed, following pPDL application during this period. Incomplete regeneration of the furcation defect in the control group was due to insufficient wound closure. These findings suggest that application of pPDL promotes cells population of the bone and root surfaces of furcation defects, and that periodontal regeneration requires a certain time period to take place. Epithelial downgrowth was noted in the furcation defect at 2 weeks after surgery in the control group. The early appearance of stable tissue on the root surface interferes with epithelial growth toward the root surface, as clearly seen in the pPDL treatment group (16).

VEGF expression level in pPDL was determined by RT-PCR. The VEGF mRNA level increased in the pPDL compared with connective tissue (control group). This study showed that growing capillaries associated with abundant fibroblasts (mesenchymal cells) invaded the wound region from surrounding bone marrow 2 weeks after surgery in the pPDL treatment group. These findings demonstrate that pPDL with a high VEGF expression level supplies abundant blood vessels to the wound. Several growth factors, such as VEGF and bone morphogenetic proteins, modulate bone formation and angiogenesis during bone wound healing. Previous studies have demonstrated that immunoreactivity for VEGF can be detected in chondrocytes and osteoblasts in the fracture callus during healing process (23, 28). A recent study demonstrated that inhibition of VEGF decreased angiogenesis, bone formation, and callus mineralization in a mouse bone injury model (25). It has been shown that VEGF is essential in the coordination of angiogenesis and bone formation, and that it may play a crucial role in bone formation and neovascularization. Therefore, these results suggest that pPDL with a high VEGF expression level supplies abundant blood vessels to the wound. Several growth factors, such as VEGF and bone morphogenetic proteins, modulate bone formation and angiogenesis during bone wound healing. Previous studies have demonstrated that immunoreactivity for VEGF can be detected in chondrocytes and osteoblasts in the fracture callus during healing process (23, 28).

Furthermore, our study detected an increase in bFGF mRNA expression level in pPDL. The bFGF is known to promote the proliferation of mesenchymal cells, including osteocytes and PDL cells, and to stimulate the osteogenic expression of bone marrow stromal cells (19). It has been reported that bFGF showed mitogenic activity on undifferentiated
mesenchymal cells, and that undifferentiated mesenchymal cells proliferated upon stimulation with bFGF (27). In our study, there were more PCNA-positive cells around the new bone tissue in the furcation defects in the pPDL treatment group than in the control group, suggesting that bFGF in the pPDL induced the proliferation of undifferentiated mesenchymal cells which then differentiated into osteoblasts and cementoblasts. Therefore, bFGF may increase the potential for desirable periodontal regeneration, mainly bone regeneration, in the furcation area. This agrees with the findings of an earlier study by Nakahara et al. (20).

The regeneration of both hard and soft tissues is essential for complete periodontal regeneration. pPDL affects several aspects of periodontal regeneration such as osteogenesis, cementogenesis, and connective tissue regeneration. None of these processes was noted in the control group. These results support the findings of our previous study showing that treatment without pPDL inhibited periodontal wound healing (12). In the present study, epithelial downgrowth occurred in the furcation defect in the control group. In contrast, pPDL treatment effectively stimulated periodontal regeneration within a short period. Therefore, this study proposes a novel therapeutic approach and a new research strategy for dental and craniofacial surgery.

In conclusion, this study found histological evidence that treatment with pPDL induced periodontal regeneration in mandibular class III furcation defects. This suggests that the healing pattern induced by this treatment is more advantageous than that of flap surgery alone.

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REFERENCES

Periodontal regeneration of furcation defect by pPDL.


