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Formation of new periodontal ligament around transplanted teeth with proliferating tissue in periodontal osseous defect under barrier membrane

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ABSTRACT

The present study evaluated whether transplanted proliferating tissue in periodontal osseous defects can form new periodontal ligament. A periodontal osseous defect was created in dogs by removing alveolar bone around the circumference of the premolar, and was covered with the membrane or without the membrane at control sites. After two weeks, the proliferating tissue in the periodontal osseous defect under the barrier membrane was excised, while at each control site, the connective tissue was dissected. The proliferating tissue and tooth were transplanted to holes drilled in the alveolar bone, while at the control sites, the connective tissue and tooth were transplanted. After one, two and four weeks, the dogs were sacrificed and examined histologically; a LightCycler was also used to measure the mRNA expression of vascular endothelial growth factor (VEGF) and alkaline phosphatase (ALP) in the transplanted tissues. VEGF and ALP mRNA expressions were significantly increased in the transplanted proliferating tissue compared with the connective tissue (control). The root transplanted bearing the proliferating tissue formed new periodontal ligaments, whereas connective tissue could be observed on the root surface in the controls. The results of this study show that transplanted proliferating tissue promotes the formation of new periodontal ligaments around the root.

The biological principle of guided tissue regeneration (GTR) involves the re-establishment of new periodontal ligaments to the root surface and the predictable regeneration of lost tissue. Previous studies on GTR have used microporous membranes to guide cells from the periodontal ligament to the covered root surface to stimulate tissue proliferation under a barrier membrane (20). Animal experiments have shown the formation of new periodontal ligaments on the surface of previously exposed roots due to GTR (5, 9, 13). The results suggest that proliferating tissue under the barrier membrane contains cells and extracellular matrix macromolecules associated with the formation of bone, cementum and periodontal ligament. Proliferating tissue under a barrier membrane with the capacity to regenerate periodontal tissue may be useful to develop treatments for the use of proliferating tissue (3, 4, 15, 21).

Amar *et al*. (4) evaluated the distribution of matrix macromolecules in proliferating tissue under the barrier membrane and observed positive reactions for major matrix macromolecules such as collagen types I, III and V, bone sialoprotein and osteonectin, which strongly suggests that this proliferating tissue provides an environment favorable for the phenotypic expression of specific periodontal tissues. Ivanovski *et al*. (10) showed that macromolecules essential for the generation of both the soft and the hard tissue of the periodontium are present in proliferating tissue under the barrier membrane, and they are distinct tissue components of periodontal tissue.

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The distribution of osteocalcin, osteopontin, and in particular bone sialoprotein among these components indicates osteogenic and cementogenic activities which are vital for periodontal regeneration.

Vascular endothelial growth factor (VEGF) has recently attracted attention as a protein that induces vascular permeability and angiogenesis (8, 14, 19). VEGF is a potent angiogenic factor which is expressed in various highly vascularized tissues. Some *in vitro* biochemical studies have shown that VEGF induces the differentiation of osteoblasts and alkaline phosphatase (ALP) activity (6, 7). Suthin *et al.* (29) reported that VEGF is involved in the regeneration of periodontal tissue by promoting calcification. These results suggest that VEGF induces the formation of hard tissue that is involved in periodontal tissue regeneration.

In this study, we transplanted proliferating tissue under a barrier membrane to holes made in the alveolar bone, expecting that this tissue might play a role in the formation of new periodontal ligaments in the wound healing process. The purpose of this study was to evaluate the possible ability of transplanted proliferating tissue to induce the regeneration of periodontal ligaments by examining the expression of VEGF and ALP using immunohistochemistry and reverse transcription PCR.

MATERIALS AND METHODS

Twenty young, healthy beagle male dogs were used for this study. In dogs under general anesthesia with ketamine hydrochloride (Ketaset) at a dosage of 10 mg/kg and xylazine (Rompum) at a dosage of 1.5 mg/kg and local anesthesia (2% Xylocaine, 1 : 8,000 epinephrine), fourth mandibular premolars ($_4P_4$) were extracted to provide space (recipient sites) (Fig. 1). This was done according to the Guidelines for the Treatment of Experimental Animals at the Tokyo Dental College. The resulting alveolar ridges were allowed to heal for 3 months.

Two weeks before experimental day 0, periodontal osseous defects were created in the mesial root on $_3P_3$ (Fig. 1). The periodontal osseous defects, from the alveolar crest to the bottom of each bone defect, were set to 5×6 mm as measured with a periodontal probe (Fig. 2). The surface of the root was thoroughly scaled and the root was planed. To prevent extending osteoblasts from the bone wall of the periodontal osseous defects, bone wax (32) (B. Braun Medical™; BRAVN Inc., Bethlehe, PA, USA) was used. The defect was covered with an e-PTFE membrane (GTAM Oval-9; WL Gore & Associates, Flagstaff, AZ, USA). The flaps were then replaced and sutured. At the control site, no membrane was used and the flaps were replaced and then sutured. At experimental day 0, the experimental site ($_3P_3$) was the proliferating tissue growing under the e-PTFE membrane (transplanted proliferating tissue) which was carefully excised, while at the control site, a split thickness flap was elevated and the tissues found in contact with the root surfaces

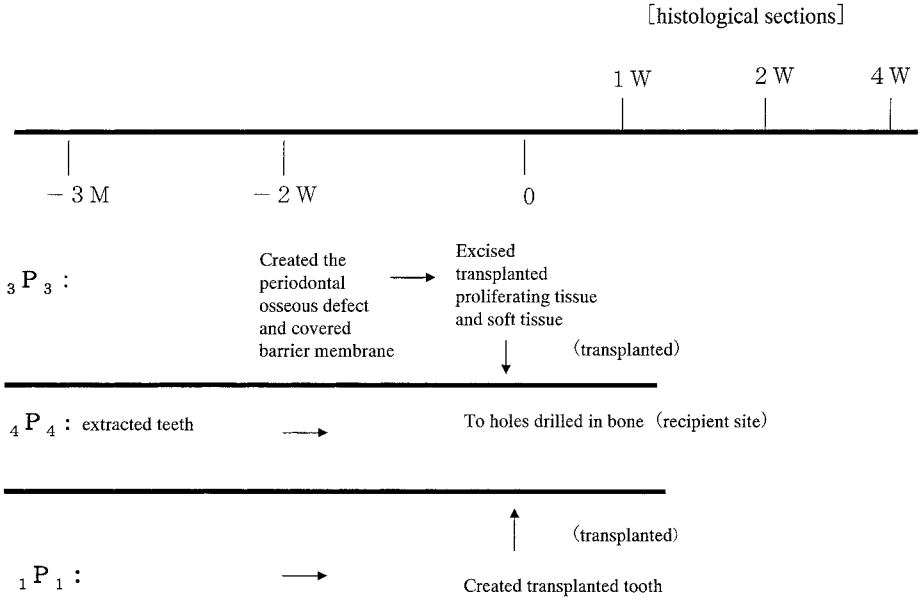

Fig. 1 Outline of the experiment

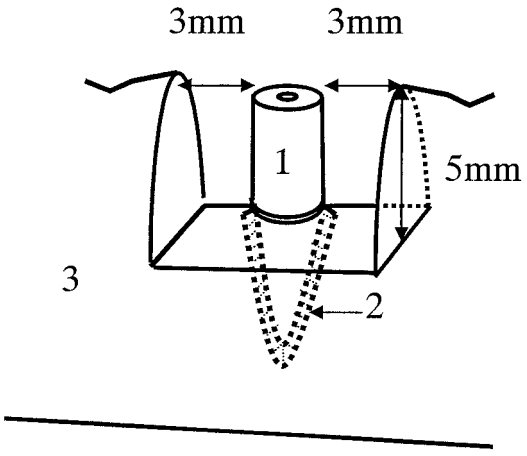

Fig. 2 Schematic drawing demonstrating the periodontal osseous defect. 1: root, 2: periodontal ligament, 3: alveolar bone

(transplanted connective tissue) were carefully dissected (Fig. 1). The crowns of the mandibular first premolars ($_1P_1$) were cut off at the level of the cement-enamel junction, and the roots of first premolars ($_1P_1$) were extracted. The roots were thoroughly scaled and planed. The transplanted root blocks ($_1P_1$) were made to 10 × 3 mm (Fig. 1). At each recipient site of endentulous posteriors on $_4P_4$, a full thickness flap was prepared (Fig. 3). A hole, from the alveolar crest ($_4P_4$) to the bottom of a hole, was set to 12 × 4 mm using implant drill (Fig. 3). At the experimental sites, the proliferating tissue was transplanted to the prepared holes of bones using a curette, after which the transplanted roots ($_1P_1$) were placed in the same holes of the bones (Fig. 3). Afterward, the defects were covered with e-PTFE membrane and the flaps were replaced and sutured. At the control site, the same transplantation method was used (Fig. 3). The same treatment was applied for the transplantation, except that the connective tissues were transplanted.

The animals were euthanized with an intravenous overdose of sodium pentobarbital at one, two and four weeks after treatment. The jaw of each animal was removed, and specimens containing the experimental areas were placed in buffered formalin. Specimens were decalicified with 10% ethylenediamine tetraacetic acid (EDTA; Wako, Tokyo, Japan). The specimens were then dehydrated in ethanol, embedded in paraffin, and serially sectioned (at 5 μm thickness) in a buccolingual orientation. The sections were then stained using hematoxylin-eosin.

Immunohistochemistry. For immunohistochemical analysis, paraffin sections (approximately 5 mm thick) were cut in the same manner as described above and were incubated in 0.3% hydrogen peroxide for 30 min at room temperature to block endoge-

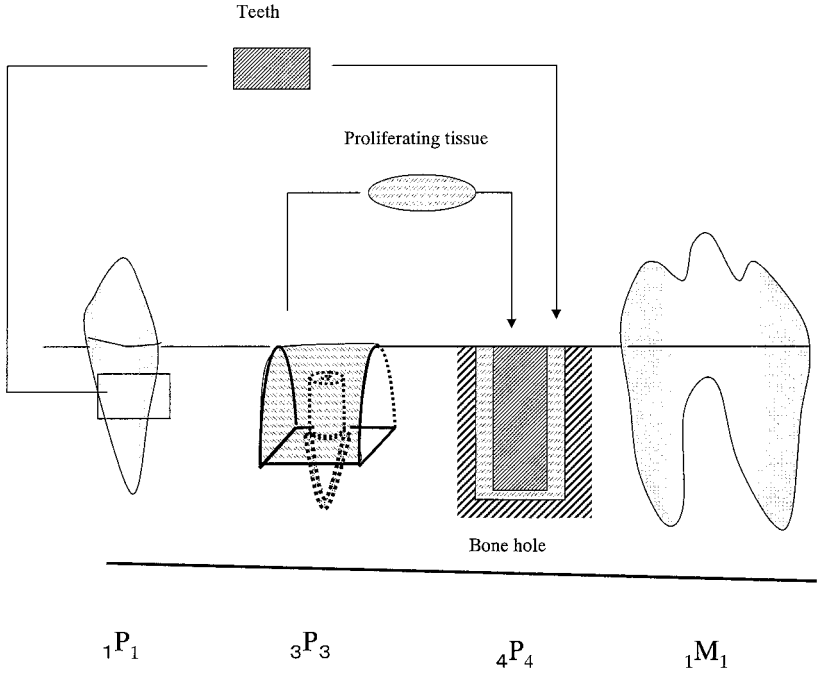

Fig. 3 Schematic drawing illustrating the experimental procedures. The proliferating tissue in the periodontal osseous defect ($_3P_3$) was excised. The proliferating tissue and tooth ($_1P_1$) were transplanted to the hole ($_4P_4$).

nous peroxidase activity after which the sections were rinsed with phosphate buffered saline (PBS) for 5 min. For antigen retrieval, sections were treated with 0.1% trypsin for 30 min at room temperature and processed using an immunoperoxidase staining kit (Histofine SAB-PO (M) kits; NICHIREI, Tokyo, Japan). The sections were incubated with mouse anti-VEGF primary antibody (V-1253; SIGMA, St. Louis, MO, USA) at a dilution of 1 : 400,000) for 12 h at 4°C. Next, the sections were incubated for 30 min each with biotinylated secondary antibody and streptavidin peroxidase reagents. The presence of peroxidase-complexes was visualized by diaminobenzidine tetrahydrochloride (0.1 mg/ml) solution with 0.01% H_2O_2 for 5 min. A brown coloration indicated a positive reaction.

Preparation of the transplanted proliferating tissue for quantitative RT-PCR using the LightCycler™. Total RNA was extracted from the samples by the acid guanidinum thiocyanate/phenol-chloroform method. The re-entry surgical procedure was performed in order to obtain biopsies for the samples (the transplanted proliferating tissue) during the 2-week period after surgery. The transplanted proliferating tissues were excised and were 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 RNAase free water, and kept at −80°C until use.

The total RNA was measured by absorbance in a 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 and $MgCl_2$: 4 μl were added to total RNA: 1 μg and the volume was adjusted to 20 μl with RNase free water. The mixed solution was used for the reverse transcriptional reaction (42°C 15 min, 99°C 5min) to synthesize cDNA. PCR was then carried out using primers for VEGF, ALP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 1). To measure mRNA levels, quantitative PCR assays were conducted with a LightCycler™ using the double-stranded DNA dye SYBR Green I (Roche Diagnostics, GmbH, Germany). Quantification was performed by comparing of the levels obtained from standardized samples. The PCR conditions used in the LightCycler™ were: 45 cycles VEGF (95°C 10 sec, 54°C 5 sec and 72°C 8 sec); 45 cycles ALP (95°C 10 sec, 57°C 5 sec and 72°C 8 sec); 45 cycles GAPDH (95°C 10 sec, 50°C 5 sec, 72°C 8 sec); melting curve analysis was also performed after the PCR amplification to confirm the absence of the primer dimer in the PCR products. The ratios of VEGF and ALP mRNA expression were corrected for the value of the housekeeping gene GAPDH. The PCR products were separated on 2% agarose ethidium bromide gels. Each PCR fragment was verified as canis VEGF: AF133248, canis ALP: AF149417, canis GAPDH: AB038240 (Nihon Gene Research Lab's Inc., Tokyo, Japan). Student-t-test was used for statistical analysis in this study.

RESULTS

mRNA expression analyses of the transplanted proliferating tissue

The expression of VEGF mRNA was examined the samples during the 2-week period after surgery by RT-PCR using a LightCycler (Fig. 4). VEGF mRNA expression was detectable in the majority of the experimental sites. VEGF mRNA expression levels were higher in the experimental sites than in the control sites, but not significant (Fig. 4). ALP mRNA expression was detectable in the majority of the experimental sites. ALP mRNA expression was detected significantly at higher levels in the experi-

Table 1 *Primer sequences and products size*

Gene	Primer sequence	Product size
VEGF	forward primer 5'-AGTTCATGGAGTACCGCG-3'	215 bp
	reverse primer 5'-CTTGAGGTTTGATCCGCAT-3'	
ALP	forward primer 5'-TCAACAGACCCTGAAATACGC-3'	202 bp
	reverse primer 5'-TCTTGGAGAGGGCCACGTAAG-3'	
GAPDH	forward primer 5'-TGGAGATGGATTCCCGTTG-3'	199 bp
	reverse primer 5'-CAACGGATTTGGCCCGTTTG-3'	

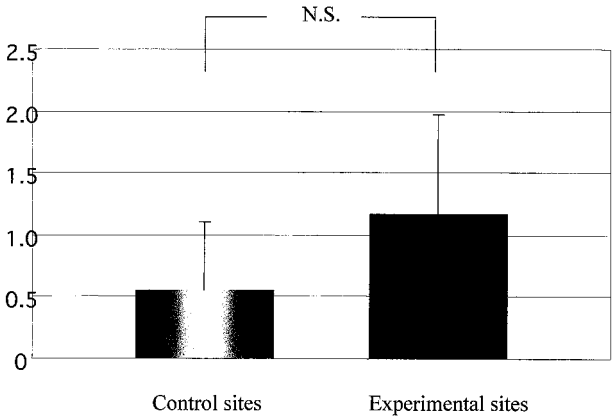

Fig. 4 The mRNA expression of VEGF in the transplanted proliferating tissue during the two week period after surgery. Data show means ± S. D. (n = 5). N.S.: not significant

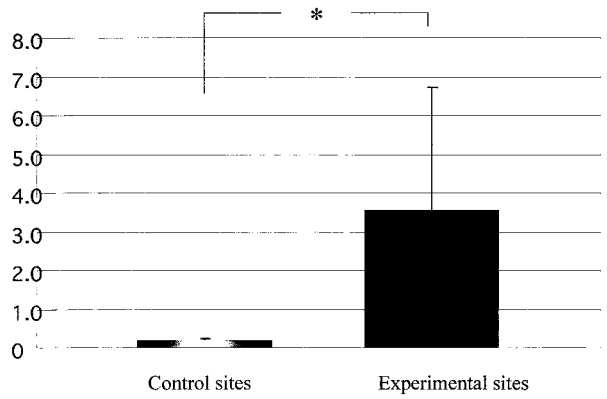

Fig. 5 The mRNA expression of ALP in the transplanted proliferating tissue during the two week period after surgery. Data show means ± S. D. (n = 5). *: significantly different from the control ($P < 0.01$)

mental sites than in the control sites (Fig. 5).

Histology

Control site: The surfaces of the transplanted roots in the control sites were covered by connective tissue from one week to four weeks after surgery (Fig. 6). This occurred as loose connective tissue or as a fibrous layer that formed a capsule. Resorption of the root surface was frequently observed. The sites of the resorption were usually occupied by connective tissue cells (Fig. 6).

Experimental site: One week after the surgery, the spaces of the bone and dentin were filled with newly-formed connective tissue that was composed of fibroblast-like cells and capillaries (Fig. 7). The numerous spindle-shaped, fibroblast-like cells were interspersed in a dense fibrillar extracellular matrix (Fig. 7). Especially, abundant spindle-shaped cells

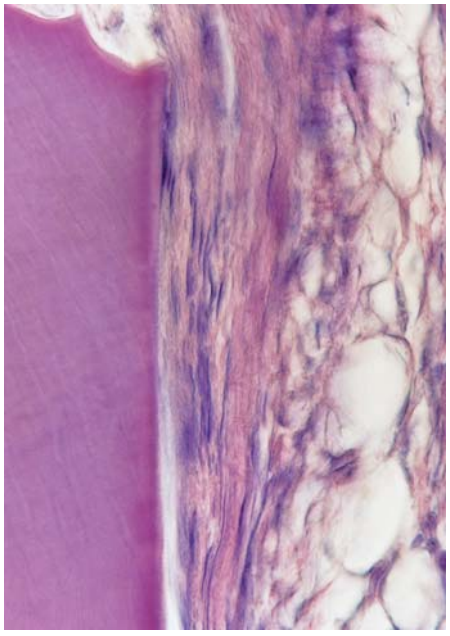

Fig. 6 Microphotograph of a transplanted root at four weeks (control site). The surface of the transplanted tooth is covered by connective tissue. (H-E stain: original magnification × 160)

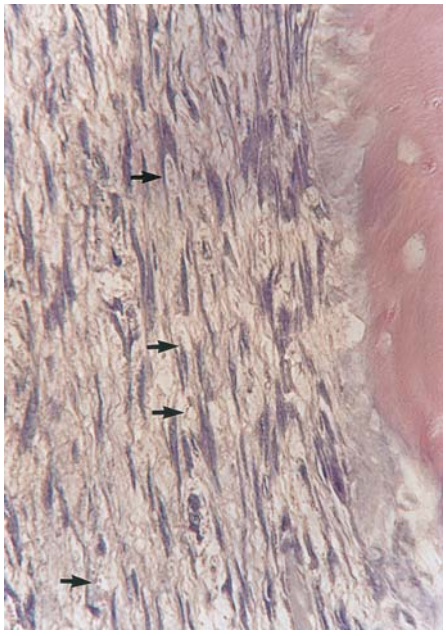

Fig. 7 Microphotograph of a transplanted tooth at one week (experimental site). Numerous spindle-shaped, fibroblast-like cells and capillaries (arrows) are seen in the newly formed connective tissue. (H-E stain: original magnification × 160)

associated with growing capillaries invaded the newly-formed connective tissue. Early bone formation was observed in the remaining alveolar bone surface. Two weeks later, the bone formation advanced parallel to the root surface (Fig. 8). As a result of the bone formation, new periodontal ligaments were formed between the root surface and the bone (Fig. 8). Further, numerous fibroblastic cells in the periodontal ligament were located in the connective tissue along the dentin surface. The dentin surface exhibited a thin layer of cementum-like deposit. The new cementum was frequently lined with cementoblast-like cells (Fig. 8). The new cementum presented as a continuous layer, covering the dentin surface to variable extents. The new bones were frequently lined with osteoblast-like cells (Fig. 8). At four weeks after the surgery, the periodontal regeneration occurred with the replacement of the fibrous connective tissue between the new bone and the dentin (Fig. 9). In those areas of the dentin where both cementum and bone were formed during healing, a periodontal ligament was reestablished. Numerous collagen fibers in the periodontal ligament were arranged in parallel to the dentin (Fig. 9). The newly formed cementum presented as a continuous thin layer and was frequently lined with cementoblasts (Fig. 9). Significant bone regeneration occurred in most of the experimental areas examined.

Immunohistochemistry

On the control sites, the immunoreactivity for VEGF was not apparent in the newly formed connective tissue including fibroblast cells. However, immunoreactivity for VEGF was detected in osteoblast cells and in endothelial cells of the newly formed bone surface. On the experimental sites, the expression of VEGF in the wound region was evaluated by immunocytochemistry. At one week after the surgery, VEGF immunoreactivity was readily detected in the newly formed connective tissue in which growing capillaries invaded the wound region (Fig. 10). The immunoreactivity for VEGF was localized in endothelial cells as well as in numerous fibroblast-like cells (Fig. 10). Immunoreactivity for VEGF was also observed in some osteoblasts of the newly formed bone surface (Fig. 10). Two weeks after the surgery, expression of VEGF diminished to undetectable levels and was not detectable in any fibroblast-like cells of the new periodontal ligament

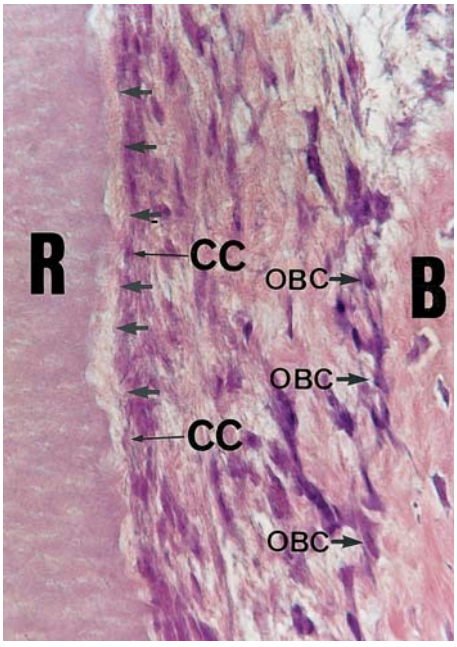

Fig. 8 Microphotograph of a transplanted tooth at two weeks (experimental site). A thin layer of new periodontal tissue is formed between the root surface (R) and the new bone (B). The continuous cementum-like deposits (arrows) are evident over the entire length of the section. CC: cementoblast-like cell, OBC: osteoblast-like cell (H-E stain: original magnification × 160)

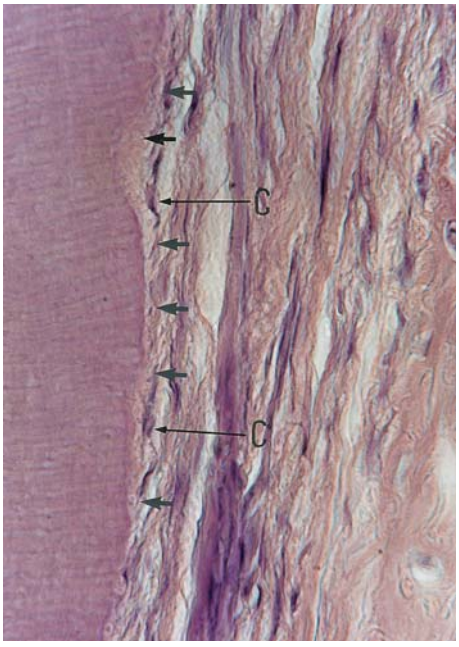

Fig. 9 Microphotograph of a transplanted tooth at four weeks (experimental site). New periodontal tissue is formed over the entire length of the section. The periodontal ligament has been re-established. The root exhibits a thin layer of new cementum (arrows). C: cementoblast (H-E stain: original magnification × 160)

(Fig. 11). However, immunoreactivity for VEGF was still detected in endothelial cells as well as in some osteoblasts of the newly formed bone surface.

DISCUSSION

In this study, the transplanted proliferating tissue in periodontal osseous defects under the barrier membrane induced the regeneration of periodontal ligaments. In all specimens of transplanted proliferating tissue, the regeneration of periodontal tissue was observed. Our previous study have demonstrated that transplanted proliferating tissue contains macromolecules associated with bone and cementum formation, while connective tissue obtained without a proliferating tissue does not express such macromolecules. This suggests that transplanted proliferating tissue has an intrinsic potential for hard tissue differentiation (cementum, alveolar bone and connective tissue fibers). The characteristics of cells and extracellular matrix observed in the transplanted proliferating tissue resembled those of periodontal tissue obtained from osseous defects treated with GTR barriers (3, 15, 21). The present histological examination of transplanted connective tissue (control) confirmed earlier studies which suggested that healing of periodontal defects achieved by conventional periodontal surgery (without a membrane) is characterized by the formation of granulation tissue lacking regenerative potential (12, 27). Transplanted proliferating tissue has a distinct ability to form hard tissue.

We showed an increase in ALP mRNA expression in transplanted proliferating tissue during the 2-week period after surgery. ALP mRNA expression significantly increased in transplanted proliferating tissue compared with connective tissue (control). ALP has been suggested to be associated with bone formation and calcification (11, 23). Robison *et al*. (24) reported that ALP is hydrolyzed to hexose monophosphate, thus increasing the local concentration of calcium phosphate. Recent studies have shown a more marked expression of ALP activity in developed periodontal membrane tissue and in periodontal tissue than in gingival tissue. Amar *et al*. (4) reported strong ALP staining of proliferating tissue obtained from periodontal osseous defects under a barrier membrane and a similar staining pattern in the continuously erupting rat incisor, i. e., the periodontal membrane was undergoing constant remodeling during the constant eruption. Such studies support the concept that the increase in ALP mRNA expression in proliferating tissue seen during the 2-week period after surgery is favorably involved in

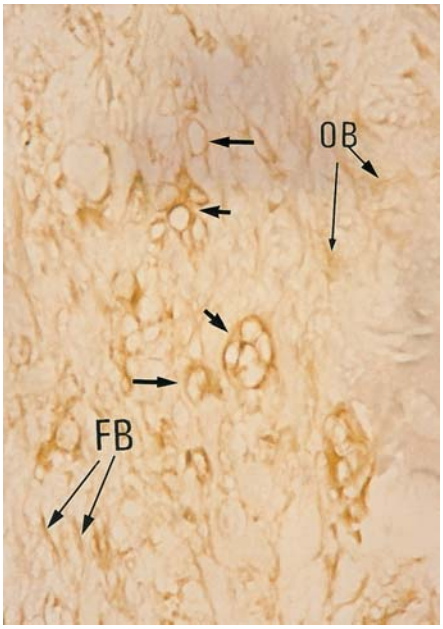

Fig. 10 Immunohistochemistry for VEGF in a transplanted tooth at one week (experimental site). Immunoreactivity for VEGF is found on some endothelial cells (arrows), osteoblasts (OB) and fibroblast-like cells (FB). (original magnification × 200)

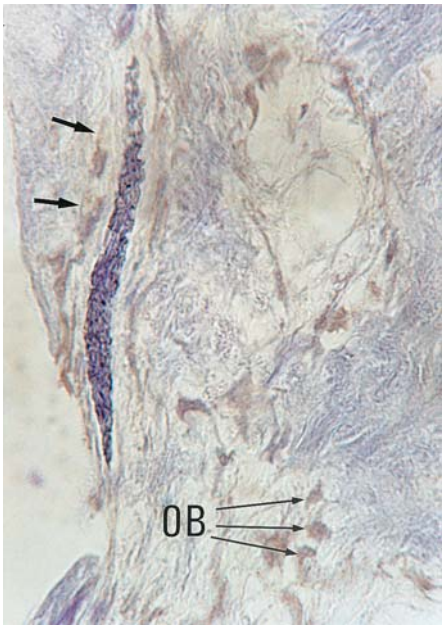

Fig. 11 Immunohistochemistry for VEGF in a transplanted tooth at two weeks (experimental site). This protein has diminished to undetectable levels. Some endothelial cells (arrows) and osteoblasts (OB) weakly express VEGF. (original magnification × 200)

hard tissue formation.

This study assessed by RT-PCR also showed VEGF expression in the transplanted proliferating tissue during the 2-week period after surgery. VEGF mRNA assessed by RT-PCR was increased in the transplanted proliferating tissue compared with the transplanted connective tissue, although the change was not significant statistically. Recent studies (22, 31) have demonstrated that VEGF and bone morphogenetic protein act as growth factors to modulate bone formation and angiogenesis during bone wound healing. Many studies (1, 2) have shown that various cytokines, transforming growth factor, and basic fibroblast growth factor regulate expression of VEGF in osteoblasts. Interestingly, a recent study (28) in a mouse bone injury model showed that inhibition of VEGF decreases angiogenesis, bone formation and callus mineralization. Suthin *et al*. (30) found that VEGF mRNA expression in human periodontal ligament fibroblasts is higher than in human gingival fibroblasts. The results of these studies suggest that VEGF mRNA expression in proliferating tissue during the 2-week period after surgery plays a role both in bone/cementum formation and in the neovascularization that occurs during the healing process for periodontal tissue regeneration.

This study showed histopathologically that VEGF was expressed in spindle-shaped cells as well as in endothelial cells of newly formed tissue after 1 week but decreased in intensity after 2 weeks. These findings are consistent with the stimulation of VEGF expression 10 days after fracture in mice (7, 31). Shweiki *et al*. (26) evaluated vascularity 7 days after fracture and also suggested the importance of VEGF in early angiogenesis. Increased VEGF activity in the early stage of bone repair has been also reported in fracture hematomas in hamsters. Those findings were consistent with our study, and strongly suggest that the expression of VEGF in spindle-shaped cells and endothelial cells may precede the formation of hard tissue in the early stage of periodontal tissue regeneration. Histopathologically, strong VEGF signals were expressed in connective tissue harvested from the transplanted proliferating tissue, particularly in numerous spindle-shaped cells and in endothelial cells, but they were negligibly expressed at the control sites. In addition, 4 weeks after transplantation, the regeneration of supporting tissue (new bone, new cementum and connective tissue fibers) was observed in all specimens in the transplanted site of proliferating tissue whereas supporting tissue was absent in the controls. Several recent studies showed VEGF mRNA expression in fibroblasts and in endothelial cells in response to hypoxia (17, 18, 26). Saadeh *et al*. (25) demonstrated increased VEGF mRNA expression in healing process of bone fractures and also in isolated osteoblasts stimulated by angiogenic growth factor. Street *et al*. (28) showed that VEGF directly regulates the differentiation of primary osteoblasts *in vitro*, and VEGF activity is indispensable for appropriate callus formation and calcification in the healing process of bone injury. Suthin *et al*. (29) observed increases in the expression of osteocalcin and osteopontin mRNAs following VEGF stimulation of cultured human periodontal ligament fibroblasts, suggesting the involvement of VEGF in calcification. These results suggest that VEGF plays a role in the hard tissue regeneration of periodontal tissue by promoting calcification.

In conclusion, this study demonstrated that transplanted proliferating tissue in periodontal osseous defects under a barrier membrane can produce the periodontal ligaments. Regenerated periodontal ligaments were not formed in connective tissue obtained from defects treated in the absence of a membrane. This suggests that transplanted proliferating tissue forms hard tissue (new bone and cementum) in the wound healing of periodontal tissue.

Acknowledgements

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