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Root Surface Conditioning with Bone Morphogenetic Protein-2 Facilitates Cementum-like Tissue Deposition in Beagle Dogs

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Running title

Cementum-like tissue formation on BMP-applied root surface.

Key words

BMP-2; Root dentin surface conditioning; cementum-like tissue; dogs

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Abstract

***Background and objective*:** Modification of the root surface may play an important role in regenerating the periodontal attachment between the root and periodontal connective tissue. We speculated that bone morphogenetic protein (BMP) application to root surface resulted in a novel attachment by cementum-like hard tissue, although non-osteogenic tissue proliferated to the root surface. The aim of this study was to examine whether BMP-2 guided cementum-like tissue deposition on a BMP-conditioned root surface.

***Material and Methods*:** Root dentin on the buccal side of 24 teeth in four beagle dogs was surgically exposed. The denuded root dentin surfaces were demineralized with EDTA and washed with saline. Subsequently, 15 µl of BMP-2 solution (loading dose; 0.4 and 1.0 µg/µl) was applied to the root dentin surface. In the control, phosphate-buffered saline was applied to the root surface. Specimens were histologically analyzed 16 weeks after surgery.

***Results*:** Formation of cementum-like tissue was frequently observed on the BMP-2-conditioned root at the coronal portion. Cellular cementum-like tissue was separated from the original cementum and encapsulated with gingival connective tissue. Cementum-like tissue formation with BMP at 1.0 µg/µl was significantly greater than those in the control and BMP at 0.4 µg/µl. Downgrowth of the junctional epithelium in the 1.0 µg/µl BMP group was significantly less than that in the control.

***Conclusions*:** Root dentin surface conditioning with BMP-2 stimulated cementum-like tissue formation and inhibited epithelial downgrowth.

Introduction

In periodontal therapy, periodontal ligament cell and osteogenic cell repopulation are theoretically considered to accelerate functional periodontal tissue reconstruction during wound healing (1). However, this is prevented by the rapid epithelial cell downgrowth along the root surface from the coronal portion in the early healing stage. Moreover, gingival connective tissue has few osteogenic cells and cannot induce new attachment, including cementum formation (2). To inhibit epithelial downgrowth and promote periodontal wound healing in case of advanced periodontal destruction, early establishment of a stable attachment between the root and gingival connective tissue should be acquired at the coronal portion.

Modification of root surface properties may play an important role in the periodontal healing process. Agents for surface demineralization could remove the surface smear layer and expose the collagen matrix when applied to an instrumented root dentin surface (3). Conditioning by these agents may provide a more biocompatible root surface for early cell migration, attachment, cell-matrix interaction, and fiber development (4-7). In addition, the extracellular matrix at the dentin surface, such as collagen fiber, is an attractive target for localizing growth factors as therapeutic agents (8). As reconstructive periodontal surgery, therefore, root surface conditioning with differentiation factors, which enhance mineralization in cells, may initiate formation of cementum, including periodontal attachment by attached non-osteogenic cells stimulated by these factors.

We hypothesized that root surface conditioning with bone morphogenetic proteins (BMPs) could guide cementum-like tissue onto the root surface. Although gingival connective tissue proliferates and/or attaches to the root surface, osteogenic modification of the root may initially acquire new and well-established attachment to gingival connective tissue, and consequently inhibition of epithelial downgrowth may be elevated. BMPs have the ability to transform pluripotent stem cells into osteoprogenitor cells (9) and induce ectopic osteogenesis (10, 11). Zaman et al. have reported that EDTA-demineralized dentin can retain BMP-2, and simulated the osteogenic activity of human periodontal ligament cells onto BMP-applied dentin (12). Miyaji et al. (13) designed a study in which dentin blocks, biomodified by BMP-2, were implanted in rat oral connective tissue where little osteogenic tissue was formed. They observed formation of new cementum-like tissue on the BMP-conditioned dentin block surfaces. In the present *in vivo* histological study in beagle dogs, we examined whether root surface conditioning with BMP induced cementum-like tissue deposition in experimental periodontal defects. We also investigated the effect of BMP application on epithelial downgrowth.

Material and Methods

Animals

Four healthy female beagle dogs, 10-12 months old, were used in this experiment. The experimental protocol (No. 5098) followed the Guidelines for the Care and Use of Laboratory Animals of the Graduate School of Medicine, Hokkaido University. Surgical procedures were performed under general anesthesia with medetomidine hydrochloride (0.1 ml/kg, Domitor, Meiji seika, Tokyo, Japan) and ketamine hydrochloride (0.1 ml/kg, Ketaral 50, Sankyo, Tokyo, Japan), and under local anesthesia with lidocaine hydrochloride (2% with 1:80,000 epinephrine, Xylocaine, AstraZeneca, Osaka, Japan).

BMP-2 construct

Recombinant human BMP-2 (98% purity) was donated by Astellas Pharma (Tokyo, Japan). The BMP-2 was diluted with phosphate-buffered saline (PBS; 5 mM, pH 7.2) to produce stock solutions of 0.4 and 1.0 μg/μl.

Surgical procedure

Following reflection of a partial thickness flap on the buccal side of the 2nd and/or 3rd maxillary and mandibular premolars, alveolar bone and periosteum to a depth of 6 mm (measured from the cemento-enamel junction) were removed using a rotating round bur under water irrigation. The root surface facing the defect was planed to remove cementum. Reference notches indicating the cemento-enamel junction and bottom of the defect were prepared on the root surfaces. Twenty-four root dentin surfaces were thus surgically exposed. Roots were then randomly assigned to each group. Subsequently, the denuded root surface was demineralized with 24% EDTA (pH 7.0) for 3 min and washed with saline. Fifteen microliters of BMP-2 solution (loading dose; 0.4 and 1.0 μg/μl) was applied to the root dentin surface by pipette and acrylic resin sponge (Fig. 1). As control, PBS alone was applied to the root surface. The flap was repositioned and securely sutured (Surgilon, Tyco Healthcare Japan, Tokyo, Japan). The animals received ampicillin sodium (300 mg/kg, Viccillin, Meiji Seika, Tokyo, Japan) daily for 3 days and a plaque control regimen with 0.5% chlorhexidine twice weekly for the entire period of the experiment.

Histological procedure

The animals were euthanized using an overdose of sodium pentobarbital (0.5 ml/kg, Nembutal injection, Abbott Laboratories, Chicago, IL, USA) following general anesthesia. Specimens were collected from the wound 16 weeks post-surgery. The tissue blocks, including teeth, bone and soft tissue, were fixed in 10% buffered formalin, decalcified in 10% formic-citric acid, and embedded along the buccolingual plane in paraffin wax. Six-micrometer-thick sections were serially prepared

and stained with hematoxylin-eosin (HE).

Histomorphometric analysis

Three HE-stained sections were taken; one was approximately from the center of the root, and the other two were 100 μm from either side of the center. The following five histomorphometric measurements were performed for each stained section using a software package (Scion image, Scion, Frederick, MD, USA):

1. Defect height: distance between the apical notch and the cemento-enamel junction.
2. Cementum-like tissue: length of newly formed cementum-like tissue on the root surface. The layer of tissue on the root surface separate from the original cementum in the serial section observed using light microscopy, and encapsulated with gingival connective tissue, was defined as cementum-like tissue.
3. Gingival connective tissue: distance between the apical extension of junctional epithelium and the coronal extension of alveolar bone or cementum.
4. Junctional epithelium: distance between the cemento-enamel junction and the apical extension of junctional epithelium.
5. Cementum-like tissue volume: Percentage of the length of newly formed cementum-like tissue in relation to the gingival connective tissue.

Statistical analysis

The means and standard deviations of each parameter were calculated for each group. Differences among the groups were analyzed using the Scheffé test. P-values < 0.05 were considered statistically significant. All statistical procedures were performed using a software package (SPSS 11.0, SPSS Japan, Tokyo, Japan).

Results

Histological observations

Due to pulp tissue exposure during the surgical procedure and tissue processing error, three specimens were eliminated from this study. Otherwise, postoperative healing was uneventful in all dogs.

In the control, gingival connective tissue was seen attached to the root surface in most areas. There was no cementum-like tissue on the root surface. Downgrowth of the junctional epithelium was frequently observed in the coronal portion. Cementum continuing from original periodontal tissue was localized in the apical portion of the defect. There was no ankylosis, but mild induction of bone was noted (Fig. 2-A).

In the BMP-applied group, the serial histologic sections showed that new cementum-like tissue with no continuation with the original cementum was deposited on the root surface (Fig. 2-C, 3-C). Cementum-like tissue was evident in four sites receiving BMP at 0.4 μg/μl (4/6) and five sites at 1.0 μg/μl (5/7). We frequently detected thick cementum-like cellular tissue with a layered structure, i.e., cement line, on the root surface conditioned with BMP at 1.0 μg/μl (Fig. 3-C). In contrast, cementum-like tissue in BMP at 0.4 μg/μl appeared as a thin layer and with an unclear cement line, indicating immature tissue (Fig. 2-C). Cementum-like deposits in the BMP-applied group was lined by cementoblast-like cells and attached to gingival connective tissue. Downgrowth of the junctional epithelium was suppressed at the coronal portion of the root surface in BMP-applied groups (Fig. 3-B).

In the apical portion of the BMP application group, alveolar bone formation had also occurred along the root surface; however, ankylosis with dentin resorption was simultaneously noted (Fig 2-B, 3-A, D). Ankylosis was evident in five defect sites receiving BMP at 0.4 μg/μl (5/6), and all roots at 1.0 μg/μl (7/7). There was little evidence of periodontal ligament formation in the BMP groups (Fig. 3-D).

Histomorphometric analysis

The length of newly formed cementum-like tissue (mm) was 0.17 ± 0.12 and 0.68 ± 0.61 in the surfaces conditioned with BMP at 0.4 and 1.0 μg/μl, respectively. Cementum-like tissue formation in the surfaces conditioned with 1.0 μg/μl BMP was significantly greater than those in the control and in the surfaces conditioned with 0.4 μg/μl BMP. In the control, no cementum-like deposit was observed. There was a BMP dose-dependent increase in the amount of cementum-like tissue. Cementum-like tissue volume in the 1.0 μg/μl BMP group extended to approximately 40%, and significant differences were seen between the control and 0.4 μg/μl BMP groups. The length of junctional epithelium (mm) was 1.43 ± 0.43, 0.91 ± 0.26 and 0.70 ± 0.38 in the control, 0.4 μg/μl

BMP and 1.0 μg/μl BMP groups, respectively. Downgrowth of the junctional epithelium in the 1.0 μg/μl BMP group was significantly less than that in the control (Table 1).

Discussion

The present study focused on cementum-like tissue deposition by root surface conditioning with BMP-2. We selected periodontal dehiscence type defects in this study, because gingival connective tissue would come in contact with the BMP-loaded root surface in the early stage of periodontal healing. Therefore, it is reasonable to evaluate whether cementum-like tissue will be formed on root covered with gingival connective tissue in this experimental model.

Histological findings in serial sections revealed that BMP conditioning of the root surface frequently caused deposition of cementum-like tissue separate from original cementum and encapsulated with gingival connective tissue. We speculate that BMP-2 directly affects the differentiation of undifferentiated cells associated with gingival connective tissue. In our previous study, cultured cells, expanded from human gingival connective tissue, had high alkaline phosphatase activity when attached to BMP-2-applied dentin surfaces (14). Furthermore, cementum-like tissue was formed on dentin blocks with BMP-2 conditioning in rat palatal connective tissue (13, 15). Our results support the notion that restoration of stable periodontal attachment can be guided on the root surface regardless of whether original periodontal ligament tissue remains. It seems likely that the only remaining cell source is the gingival connective tissue when the surgical flap is reattached to the instrumented root surface in case of advanced periodontitis.

The amount of cementum-like tissue formation should be augmented in BMP conditioning therapy for a more stable attachment apparatus. Takita et al. (16) reported that combined application of fibroblast growth factor 2 (FGF2) stimulated BMP-induced osteogenesis in rats. In regenerative therapy, it is well known that tissue regeneration can be promoted using a viable cell transplantation method (17, 18). In particular, cell sheets are advantageous, as a large number of cells can be easily transplanted. Further, cell sheets have the property of rapid adhesion to surrounding tissue (19). Therefore, BMP will promote cementum-like tissue deposition in various combinations with other growth factors and osteogenic cell sheet transplantation to the root surface.

Epithelial downgrowth in the BMP group was significantly less than that in the control, suggesting that downgrowth of junctional epithelium along the root surface was inhibited by attachment formation with cementum-like tissue at the coronal portion. However, we also observed that the apical end of the epithelial cell downgrowth did not necessarily fit the coronal end of the cementum-like tissue. Shipley et al. (20) reported that transforming growth factor (TGF) -beta, which is a peptide growth factor closely related to BMPs, inhibited proliferation of epithelial cells. In the present study, inhibition of the long junctional epithelium formation may relate to the additional action of BMP-2 on epithelial cells.

Consistently, we found alveolar bone induction by BMP-2 treatment. It was suggested that alveolar bone formation was augmented by BMP-2 released from the root dentin surface in this BMP

conditioning therapy. The present examination also revealed that ankylosis was induced in BMP-applied groups. Many studies using BMP-2 in various biological scaffolds found evidence of ankylosis on part of the root surface with bone regeneration in periodontal defects (21-24). In periodontal regenerative therapy, ankylosis should be inhibited by regeneration of both alveolar bone and periodontal ligament simultaneously. A previous study showed that BMP-2 had low proliferative activity on human periodontal ligament cells *in vitro* (12). From these findings, we speculate that BMP-2 abundantly localized on root dentin surfaces suppresses the proliferation of the periodontal ligament cells onto the root surface in the healing process. To prevent ankylosis in this system, additional methods will be necessary for accelerating the bioactivities of periodontal ligament cells at the apical site of the defect. Aberration resulting from BMP-2 use is an important point to be verified in the future.

In conclusion, root surface conditioning with BMP-2 promoted cementum-like tissue deposition and inhibited epithelial downgrowth. These findings suggest that BMP application to the root surface may allow periodontal attachment reestablishment in case of advanced periodontal destruction, regardless of the source of the cells which are provided to the root surface.

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Table 1. Histomorphometric analysis at 16 weeks after surgery (mean ± SD).

	Control (n=8)	0.4 µg/µl BMP (n =6)	1.0 µg/µl BMP (n = 7)
Cementum-like tissue (mm)	0.00 ± 0.00	0.17 ± 0.12	0.65 ± 0.61*†
Gingival connective tissue (mm)	2.50 ± 0.84	2.02 ± 0.67	1.57 ± 0.66
Junctional Epithelium (mm)	1.43 ± 0.43	0.91 ± 0.26	0.70 ± 0.38*
Cementum-like tissue volume (%)	0.0 ± 0.0	8.1 ± 5.5	39.0 ± 31.9*†

* Statistical difference compared to control group ($p < 0.05$).

† Statistical difference compared to 0.4 µg/µl BMP application group ($p < 0.05$).

Figure legends

Figure 1

Demineralized root surface conditioned with BMP-2 solution.

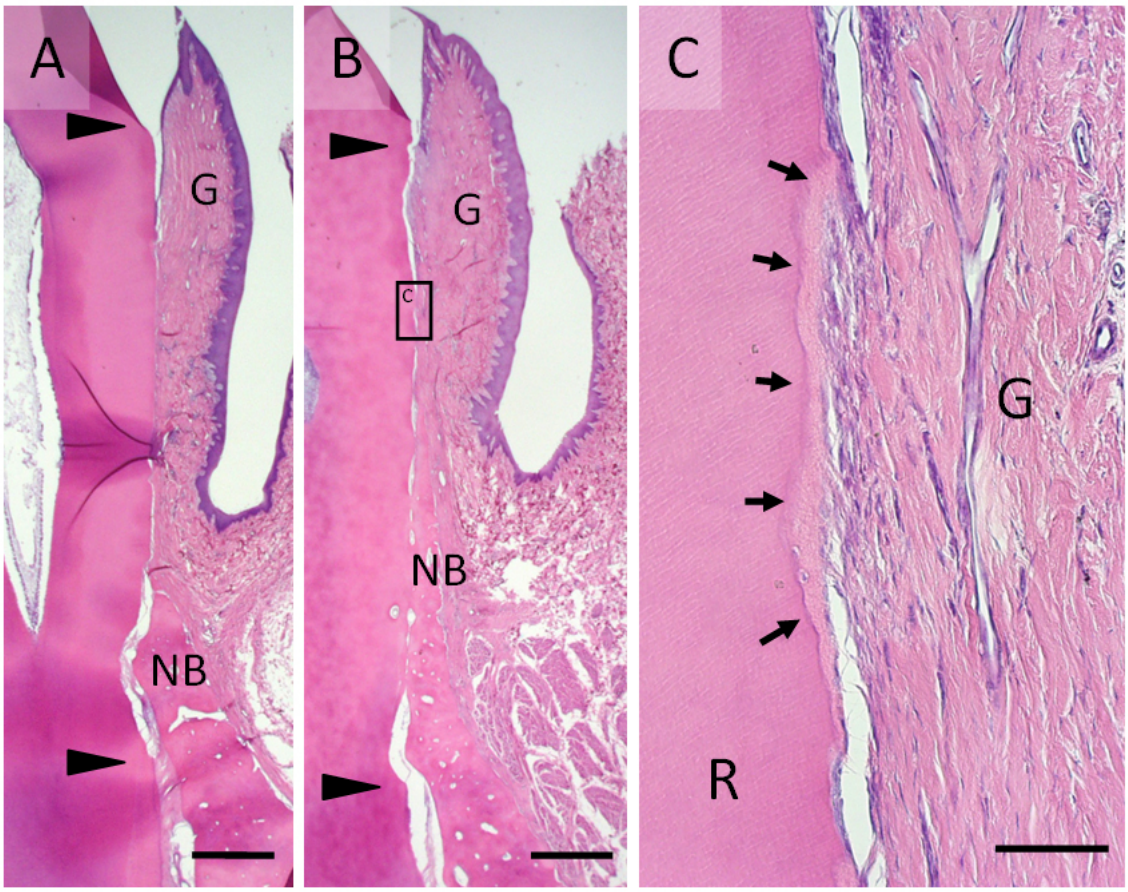

Figure 2

A) Histological findings in control. Gingival connective tissue was attached to the root surface without cementum formation. Epithelial cell downgrowth was frequently observed in the coronal portion. New bone induction was demonstrated only around the apical notch. B) Histological findings in BMP at 0.4 µg/µl. The root surface was covered by gingival connective tissue and newly formed alveolar bone. Epithelial cell downgrowth was localized at the coronal portion of the root surface. C) Higher magnification of the framed area (c) in B. Thin cementum-like tissue (arrows) separate from the original cementum was deposited on the root surface covered by gingival connective tissue. (R, root; NB, new bone; G, gingival connective tissue; HE staining; scale bars: A and B = 1 mm; C = 100 µm.)

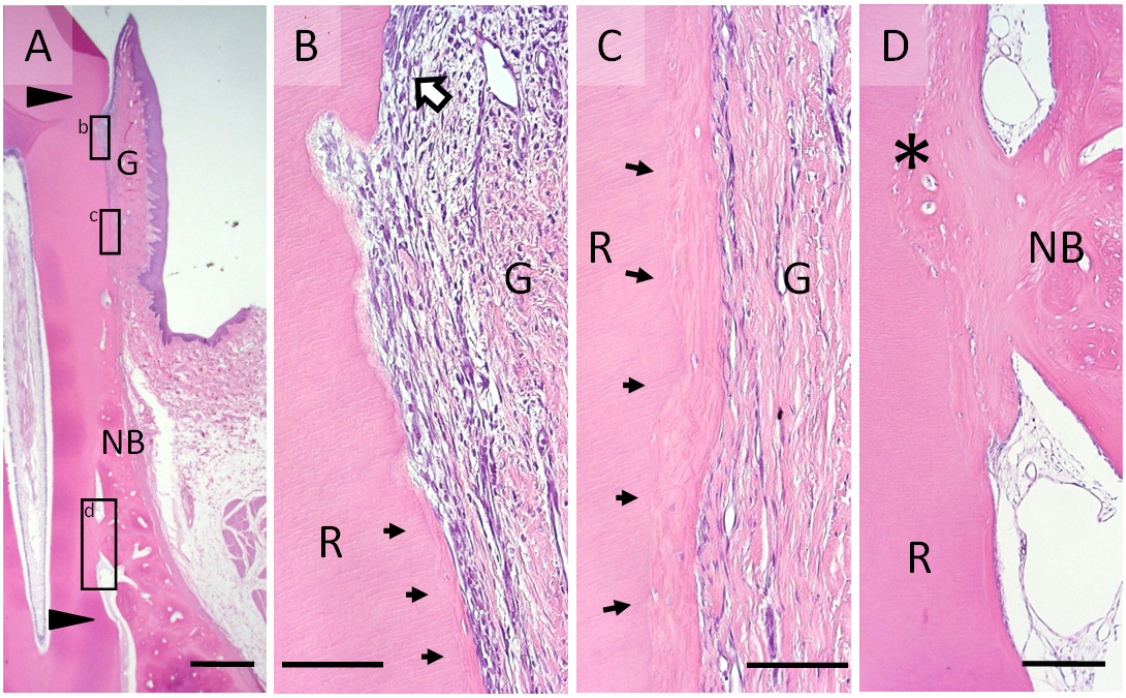

Figure 3

Histological findings in the BMP at 1.0 µg/µl group. A) Gingival connective tissue attached to the root surface, and new bone formation was induced at the apical site. B) Higher magnification of the framed area (b) in A. Epithelial cell downgrowth was localized at the coronal notch (white arrow), and cementum-like tissue (arrows) was detected on the BMP-conditioned root surface. C) Higher magnification of the framed area (c) in A. Thick layered and cellular cementum-like tissue (arrows) divided from original cementum was frequently detected on the root surface. Cementum-like tissue was encapsulated with gingival connective tissue. D) Higher magnification of the framed area (d) in A. Ankylosis (star) was frequently observed at the apical portion. (R, root; NB, new bone; G, gingival connective tissue; HE staining; scale bars: A = 1 mm; B and C = 100 µm; D = 200 µm.)