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Dentin resorption and cementum-like tissue formation by BMP application

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Running title

Tissue reactions on BMP-applied dentin.

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Key words

BMP (bone morphogenetic protein)

Dentin

Resorption

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Abstract

Objective: This study aimed to examine the effect of bone morphogenetic protein-2 (BMP-2) application on dentin resorption and cementum-like tissue formation at the dentin surfaces.

Background: Recent studies showed that BMP-2 stimulated the mineralization and osteoclast differentiation. Osteoclastic resorption by BMP-2 application may play an important role in the regulation of new cementum-like tissue formation on the dentin surfaces.

Methods: Seventy-two flat dentin blocks were prepared from rat roots, treated with 24 % EDTA, applied with 0, 100, and 400 µg/ml BMP-2, and labeled as groups 0, 100, and 400. The dentin blocks were then implanted into palatal connective tissue of rats, and specimens were prepared at two, four and eight weeks after surgery for histologic and histomorphometric analyses.

Results: BMP-2 caused a dose-dependent increase in dentin resorption by osteoclastic cells. New cementum-like tissue was randomly formed on parts of the non-resorbed and resorbed dentin surfaces in groups 100 and 400. Dentin resorption in groups 100 and 400 was significantly greater than group 0 ($p<0.01$). However, at eight weeks new cementum-like tissue formed in 41.8% of group 100, as compared to 16.2% in group 400 ($p<0.05$).

Conclusion: Dentin resorption was stimulated by a high dose of BMP-2, and cementum-like tissue was induced by a low dose of BMP-2, effectively suggesting that BMP-2 application in an appropriate dose to a dentin surface may enhance periodontal regeneration.

Introduction

Various growth factor therapies have been developed for periodontal regeneration in animal models. Specifically, bone morphogenetic protein-2 (BMP-2) promoted the mineralization of non-osteogenic cells (1-3), ectopic bone formation (4-6), and periodontal regeneration (7-10). Miyaji *et al.* (11) designed a study wherein BMP-2 was applied to dentin blocks that were then transplanted into rat connective tissue where there was little osteogenic tissue. This resulted not only in the formation of new cementum-like tissue on the dentin surfaces, but also the dentin in the resorbed area with multinucleated cells and/or deposition of new cementum-like tissue was displayed frequently.

Recent studies using dentin pit resorption assay *in vitro* have shown that BMP-2 significantly stimulated osteoclast differentiation and osteoclastic resorbing activity (12, 13). In the mineralized tissue field, it is known as a coupling process wherein osteoblasts and osteoclasts conserve the same spatial and temporal connection between each other (14). In periodontal wound healing in animals, newly mineralized tissue was frequently formed on the resorbed root surface areas (15, 16). Therefore, osteoclastic resorption by BMP-2 stimulation may play an important role in the regulation of new cementum-like tissue formation. To enhance the hard tissue induction activity on the dentin surface site, it is necessary to investigate the interaction between BMP-2-related dentin resorption and cementum-like tissue induction.

Thus, we prepared the dentin blocks applied with graded doses of BMP-2, and these were implanted in rat palatal connective tissue. In this model, we examined the BMP-2 dose-related tissue responses at the dentin surface site histologically and histomorphometrically.

Material and Methods

Preparation of Dentin Blocks

Seventy-two flat dentin blocks (size 1 × 1 × 0.3 mm) were prepared from rat roots, treated for 3 minutes with 24% EDTA at pH 7.0, and incubated in 1000 U/ml penicillin and 1000 µg/ml streptomycin overnight. Dentin blocks were then assigned to three groups according to BMP-2 application, i.e., groups 0, 100, and 400 were immersed in 0, 100, and 400 µg / ml recombinant human BMP-2 (Astellas Pharmaceutical Co. Ltd., Japan) in Phosphate buffered saline (PBS, 5mM, pH 7.2) for 10 min, respectively. Following BMP-2 application, the overflow of the BMP-2 solution was removed by gauze.

Surgical Procedures

Seventy-Two Wistar male rats (10 weeks old) were used in this experiment in accordance with the guide for the care and use of laboratory animals, Graduate School of Dental Medicine, Hokkaido University. The rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (Nembutal injection, 30 mg/kg body weight; Abbott Laboratories, USA). Partial thickness flaps of the masticatory mucosa of the hard palate were elevated, a BMP-2-applied dentin block was implanted into the connective tissue, and the flaps were repositioned and tightly sutured.

Histological Procedures

Specimens were prepared at two, four and eight weeks postsurgery. The dentin blocks with surrounding tissues were excised, fixed in 10% formalin, decalcified in 10 % EDTA, and embedded in paraffin. Serial five µm thick sections were prepared in frontal plane and stained by hematoxylin-eosin (H.E) and tartrate-resistant acid phosphatase (TRAP) staining. Immunostaining of osteocalcin was done by the use of a streptavidin-biotin staining kit (Histofine, Nichirei Co., Japan) and bovine monoclonal anti-osteocalcin (1:200, Takara Bio Inc., Japan). Dewaxed paraffin sections were incubated with 10% rabbit serum to block non-specific reactions and subsequently with an optimal solution of a primary antibody for osteocalcin for 24 hrs at 4 °C. The sections were then incubated with biotinylated secondary antibodies for 10 min, and then treated with 3, 3'- diaminobenzidine. As a negative control, sections were stained after replacing the primary antibody with PBS.

Histomorphometric Analysis

Three H-E staining sections were taken, from approximately the center of the dentin block and the others from 200 μm to both sides of center. The following four measurements were performed for each staining section using NIH Image software (National Institute of Health, USA).

-Number of TRAP positive cells: ratio of TRAP positive cells *per* total dentin block surface length.

-Length of resorbed dentin surface: the percentage of the resorbed dentin surface length to the total dentin block surface length

-Area of resorbed dentin: the percentage of the resorbed dentin area to the total dentin block area

-Length of new cementum-like tissue: the percentage of the newly formed cementum-like tissue length to the total dentin block surface length.

Statistical differences in each group were analyzed using Kruskal-Wallis and Mann-Whitney *U* test with Stat View[®] (Abacus Concepts Inc., USA).

Results

Histological Observations

In group 0, there were few signs of dentin block resorption. No cementum-like tissue formation was observed on the dentin surfaces at any experimental stage. (Fig. 1 A).

In group 100, distinct dentin resorption and cementum-like tissue deposition were demonstrated (Fig. 1 B-D). Cementum-like tissue layer was thin with irregular thickness and included cementocyte-like cells typically, whereas there was no collagen fibers insertion such as Sharpey's fibers and no other periodontal ligament-like structures. At two weeks, new cementum-like tissue was formed on parts of the non-resorbed and resorbed dentin surfaces randomly environed with numerous osteoblastic and osteoclastic cells (Fig. 1-B). At four weeks, the resorbed dentin areas were frequently lined by osteoblastic cells with new cementum-like tissue deposits. (Fig. 1-C). At eight weeks, osteoclastic and osteoblastic cells were rarely demonstrated around the dentin, and newly formed cementum-like tissue was evident and lined by fibroblastic cells (Fig. 1-D).

In group 400 at two weeks, not only the formation of cementum-like tissue but also positive dentin resorption by osteoclastic cells were shown on the dentin surface sites. However new cementum-like tissue was poorly formed both on the non-resorbed and resorbed dentin (Fig 2-A). In the four-week specimens, dentin resorption was frequently demonstrated and resorbed areas were composed of some resorption lacunae associated with TRAP positive cells (Fig 2-B, 2-D). At eight weeks, few osteoclastic cells and numerous osteoblastic cells were displayed on the dentin. Resorption lacunae were partially replaced by new cementum-like tissue (Fig 2-C). Osteocalcin was noted around the lining osteoblastic cells at the surface of the newly induced cementum-like tissue at each experimental period (Fig 2-E).

Histomorphometric analysis

Significantly more TRAP cells were counted in group 400 compared to groups 0 ($p<0.05$) and 100 ($p<0.01$) at four weeks (Fig 3-A).

The lengths of dentin resorption in groups 100 and 400 was significantly greater compared with the group 0 ($p<0.01$) at all stages. Resorbed dentin surface lengths in groups 100 and 400 extended to approximately 40% of the dentin surface, and no

significant differences were seen between both groups (Fig 3-B) at any of the experimental points. On the other hand, the dentin resorbed areas in group 400 were significantly higher than that in groups 0 ($p<0.01$) and 100 ($p<0.05$) at all stages (Fig 3-C).

In eight-week specimens it was observed that new cementum-like tissue formed in 41.8% in of group 100, as compared to 16.2% in group 400 ($p<0.05$). In group 0, no cementum-like tissue formation occurred (Fig 3-D).

Discussion

The present study focused on dose-related morphological tissue reactions at the BMP-2-applied dentin surfaces.

Ordinarily BMP-2 solution is administered by a carrier such as a collagen sponge which has a unique quality as a drug slow-release system in periodontal regenerative therapy (8-10). However, in the present study, it was unnecessary to maintain the release of BMP-2 for a long term. Due to this the BMP-2 application system was aimed at guiding the localized deposition of new hard tissue to the dentin surfaces early in the periodontal regenerative processes. This easy method in which root surfaces are conditioned by both EDTA and BMP-2 without biological carriers may be widely selected for future clinical trials.

Osteogenic and osteoclastic differentiations were affected by variations in dose of BMP-2 (1, 12). However, there has been no research on the relationship between BMP-2 dosage and dentin resorption *in vivo*. In this study, in group 400, the TRAP positive cell numbers at four weeks were significant compared to group 100, while the data of group 400 for the dentin resorbed area displayed consistent high levels and differed significantly compared to group 100. These results suggest that a relatively high dose of BMP-2 may preserve a long term high level of resorbing activity in the osteoclastic cells and stimulate osteoclastic cell formation, in accordance with previous *in vitro* findings (13). The length of dentin resorption was also analyzed and it showed no significant difference between groups 100 and 400. The length of dentin resorption may be irrelative to the dose of BMP-2 applied to the dentin surfaces.

Histological findings demonstrated that new cementum-like tissue formed on resorbed and/or non-resorbed dentin surfaces by application with BMP-2. From this evidence, we speculated that there are two different biological mechanisms during the process of cementum-like tissue formation caused by BMP-2. Some investigators have indicated that BMP-2 enhances osteogenic differentiation of undifferentiated mesenchymal cells and various osteoblastic properties in immature osteoblastic cells (1-3). Thus, BMP-2-applied dentin may have the potential of hard tissue formation in connective tissue resulting from osteoblastic differentiation of undifferentiated mesenchymal cells by biological signal(s) directly. On the other hand, formation of mineralized tissue was dependent on initial resorption of the denuded root surface in periodontal wound healing (16). Following early healing of periodontal tissue, a

remodeling process with osteoclastic resorption and deposition of repair cementum was seen at the root surface (17). Further, Gong *et al.* (18) reported that bone maturation was not observed in the long term by bisphosphonate administration which suppressed resorption by osteoclasts during ectopic bone formation with BMP application, suggesting that osteoclastic resorption may participate in hard tissue maturation in a coupling phenomenon. As BMP-2 stimulates TRAP positive cell formation (11, 19), additional deposition of new cementum-like tissue associated with resorbed dentin may have resulted in osteoclastic cell-based remodeling at the BMP-2-applied dentin.

Previous studies have appeared demonstrating the dose-dependent effect of BMP-2 on the activity of calcification at the BMP-treated dentin surfaces *in vitro* (20, 21). It is interesting to note in this study, however, that more cementum-like tissue was formed in group 100 compared to group 400. Some investigators reported (13, 22, 23) that *in vitro* bioactivities of cells were accelerated by growth factors such as BMP, PDGF, and TGF- β with optimal concentration, but inhibited with upper concentration. Thus, in the current experiment, application at BMP-2 overdose for rats may have the inhibitive action for cementum-like tissue induction with osteoblastic differentiation or coupling phenomenon selectively. In addition, tissue reactions *in vivo* at the dentin surfaces may be conceivably regulated by variations in the dosage of BMP-2 with additional conditions, such as cell-to-cell and/or cell-to-extracellular-matrix interactions and various biological inhibitors. Further studies are needed to elucidate these mechanisms.

In conclusion, this study elucidated that the dentin resorption by osteoclastic cells was facilitated by the application of a high dose BMP-2, and cementum-like tissue formation was promoted effectively with a relatively low dose BMP-2. Application of BMP-2 to dentin surfaces under appropriate dose control may enhance new predictable cementum-like tissue formation on the surfaces and selective periodontal regeneration.

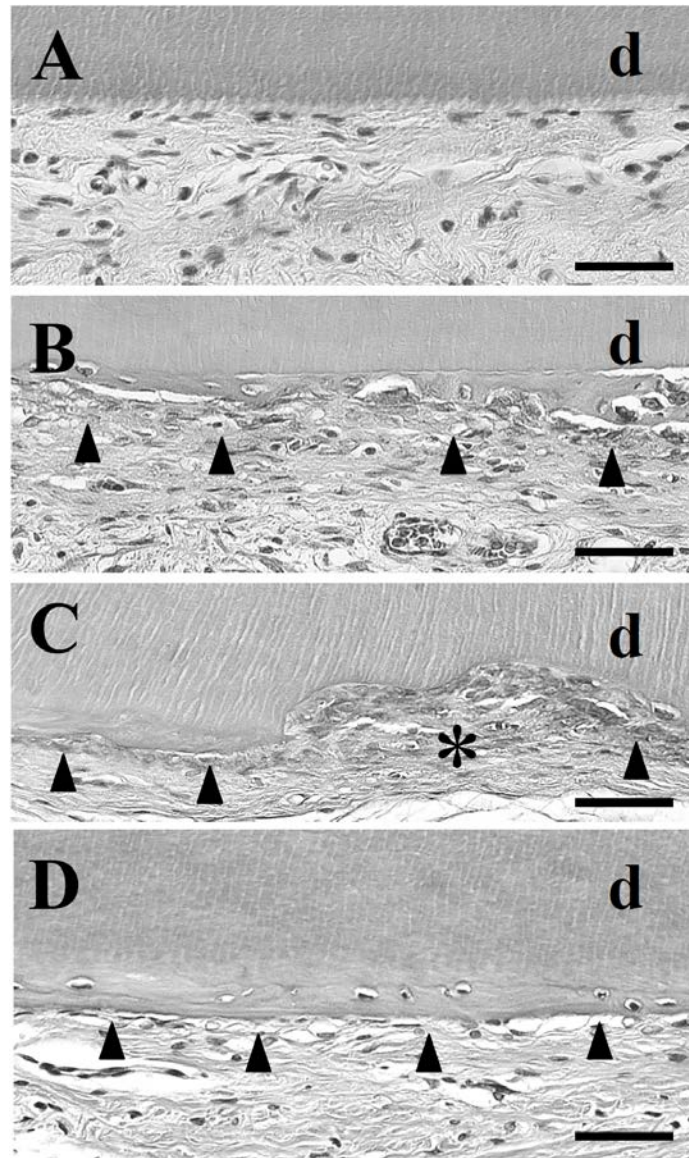


Figure. 1

(A-D) HE staining. (A) group 0 at 8th week, (B) group 100 at 2nd week, (C) group 100 at 4th week, (D) group 100 at 8th week. In group 0, fibroblastic cells and no cementum-like tissue formation were observed on the dentin (d) surfaces. In group 100, new cellular cementum-like tissue (arrowheads) was demonstrated on dentin (d) surface. Frequently resorbed dentin areas were lined by osteoblastic cells with new cementum-like tissue deposition (asterisk). Scale bar, 50 μ m.

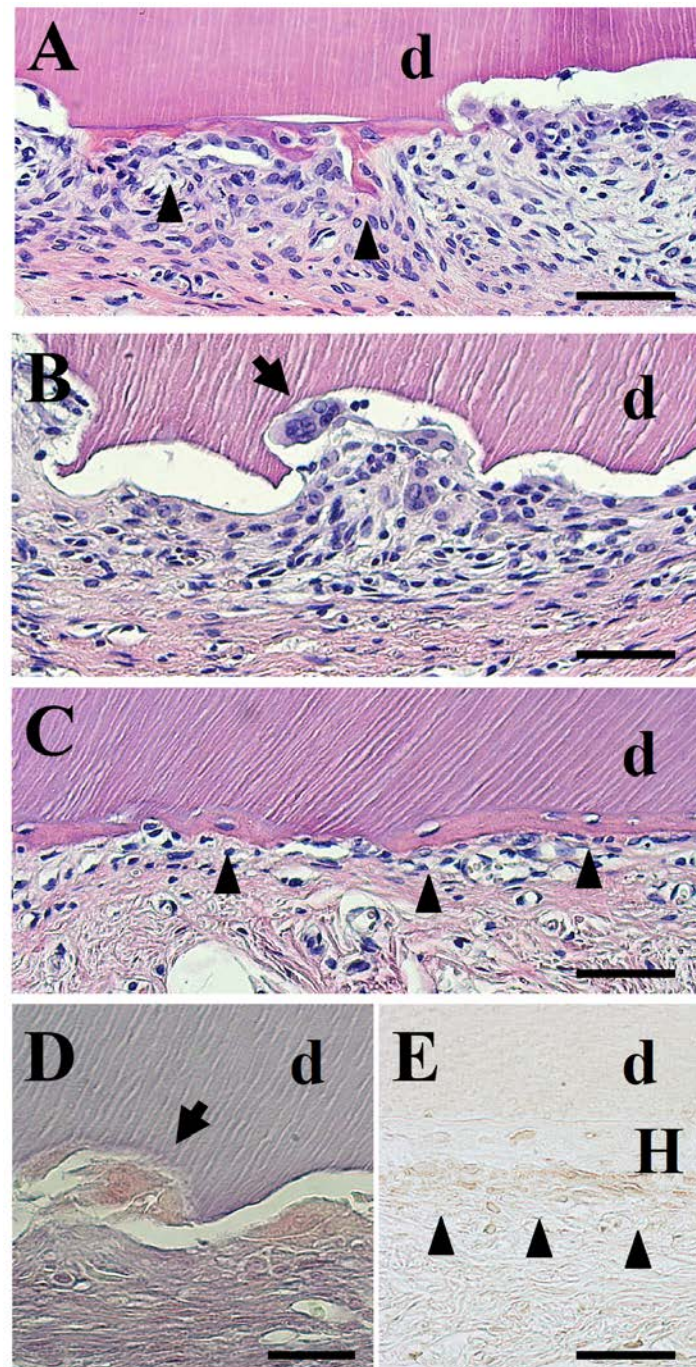


Figure. 2

(A-C) HE staining. (A) group 400 at 2nd week; (B) group 400 at 4th week; (C) group 400 at 8th week; Dentin resorption by multinucleated cells (arrows) were shown on the dentin (d) surfaces. New cementum-like tissue (arrowheads) had formed on dentin surfaces. (D) TRAP staining at four weeks. TRAP positive cells (arrows) were observed in the resorption lacunae. (E) Osteocalcin immunolocalization at eight weeks. Osteocalcin was localized on the osteoblastic cells. Scale bar, 50 μ m.

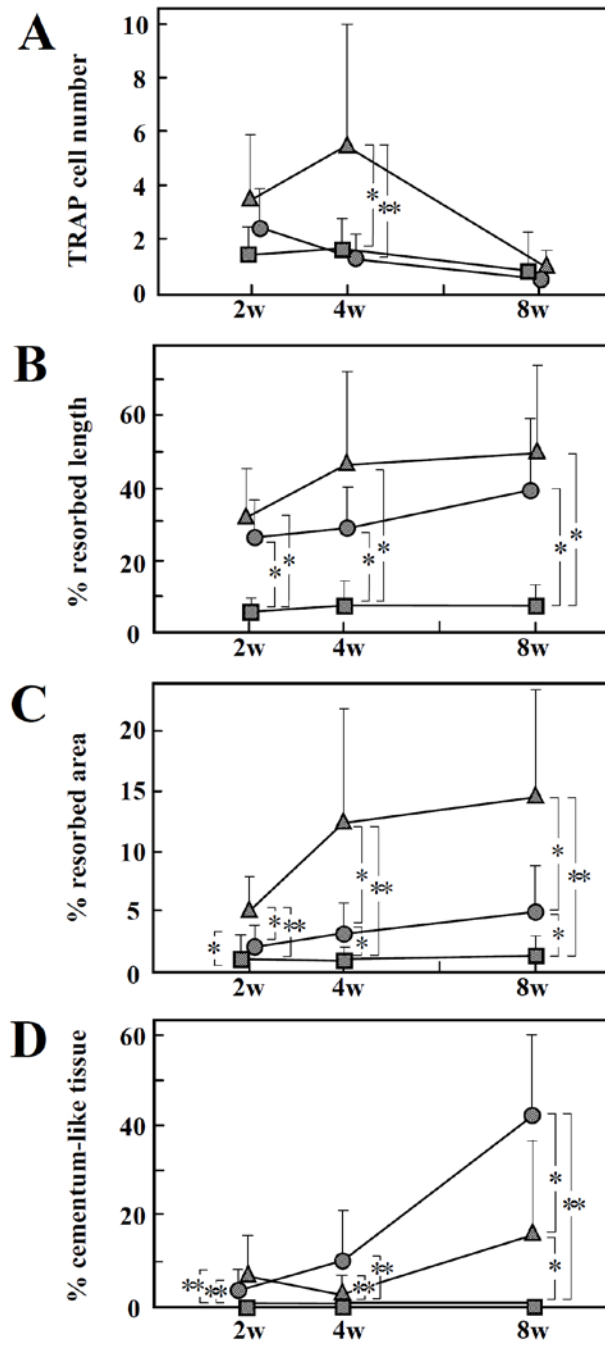


Figure 3.

Effects of BMP-2 application on (A) number of TRAP positive cells, (B) length of resorbed dentin surface, (C) area of resorbed dentin, (D) length of new cementum-like tissue in groups 0 (■), 100 (●), and 400 (▲). The results are expressed as the mean ± SD. *: $p < 0.05$, **: $p < 0.01$. Statistical differences in each group were analyzed using Kruskal-Wallis and Mann-Whitney U test with Stat View® (Abacus Concepts, Inc., USA).

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