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Dentin resorption and cementum-like tissue formation by bone morphogenetic protein application

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Background and Objective: Recent studies have shown that bone morphogenetic protein-2 (BMP-2) stimulates 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. Therefore, this study aimed to examine the effect of BMP-2 application on dentin resorption and cementum-like tissue formation at the dentin surfaces.

Material and Methods: Seventy-two flat dentin blocks were prepared from rat roots and treated with 24% EDTA. Each block was assigned to group 0, group 100, or group 400, and immersed correspondingly in 0, 100, or 400 μg/ml BMP-2. The dentin blocks were then implanted into palatal connective tissue of rats, and specimens were prepared 2, 4 and 8 wk 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 nonresorbed 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 8 wk, new cementum-like tissue formed in 41.8% of group 100, as compared with 16.2% of 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, at an appropriate dose, to a dentin surface may enhance periodontal regeneration.

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Various growth factor therapies have been developed for periodontal regeneration in animal models. Specifically, bone morphogenetic protein-2 (BMP-2) has been found to be involved in promoting the mineralization of nonosteogenic 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 a 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, this 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 implanted these in rat palatal connective tissue. Using this model, we examined, histologically and histomorphometrically, the BMP-2 dose-related tissue responses at the dentin surface site.

Material and methods

Preparation of dentin blocks

Seventy-two flat dentin blocks (size $1 \times 1 \times 0.3$ mm) were prepared from rat roots, treated for 3 min with 24% EDTA (pH 7.0), and incubated in 1000 U/ml penicillin and 1000 μg/ml streptomycin overnight. Dentin blocks were then assigned to one of three groups (0, 100 or 400), according to BMP-2 application, and processed as follows: the blocks of groups 0, 100, and 400 were immersed, respectively, in 0, 100, or 400 μg/ml recombinant human BMP-2 (Astellas Pharma Inc., Tokyo, Japan), in phosphate-buffered saline (PBS; 5 mM, pH 7.2) for 10 min. Following BMP-2 application, the overflow of the BMP-2 solution was removed by gauze.

Surgical procedures

Seventy-two Wistar male rats (10 wk 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, Abbott Park, IL, 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 2, 4 and 8 wk postsurgery. The dentin blocks with surrounding tissues were excised, fixed in 10% formalin, decalcified in 10% EDTA, and embedded in paraffin. Serial, 5-μm-thick sections were prepared in frontal plane and stained with hematoxylin and eosin (H&E) and tartrate-resistant acid phosphatase (TRAP). Immunostaining of osteocalcin was carried out by the use of a streptavidin–biotin staining kit (Histofine; Nichirei Co., Tokyo, Japan) and bovine monoclonal anti-osteocalcin (1 : 200; Takara Bio Inc., Otsu, Japan). Dewaxed paraffin sections were incubated with 10% rabbit serum to block nonspecific reactions and subsequently with an optimal solution of a primary antibody for osteocalcin for 24 h at 4°C. The sections were then incubated with biotinated secondary antibodies for 10 min, and then treated with 3,3′-diaminobenzidin. As a negative control, sections were stained after replacing the primary antibody with PBS.

Histomorphometric analysis

Three H&E-staining sections were taken, one from approximately the center of the dentin block and the other two from 200 μm to either side of the center. The following four measurements were performed for each staining section using NIH Image software (National Institute of Health, Bethesda, MD, 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; and
- 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*-tests with Stat View® (Abacus Concepts Inc., Berkeley, CA, 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. 1A).

In group 100, distinct dentin resorption and cementum-like tissue deposition were demonstrated (Fig. 1B–D). The cementum-like tissue layer was thin, with irregular thickness, and typically included cementocyte-like cells. There was no insertion of collagen fibers, such as Sharpey's fibers, and no other periodontal ligament-like structures. At 2 wk, new cementum-like tissue was formed on parts of the nonresorbed and resorbed dentin surfaces randomly environed with numerous osteoblastic and osteoclastic cells (Fig. 1B). At 4 wk, the resorbed dentin areas were frequently lined by osteoblastic cells with new cementum-like tissue deposits (Fig. 1C). At 8 wk, osteoclastic and osteoblastic cells were rarely demonstrated around the dentin, and newly formed cementum-like tissue was evident and lined by fibroblastic cells (Fig. 1D).

In group 400 at 2 wk, 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

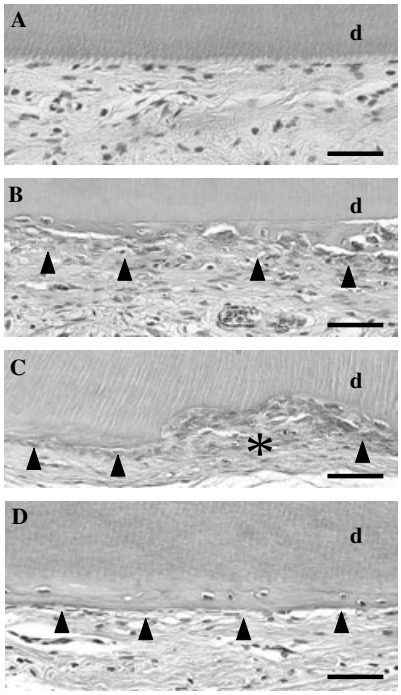

Fig. 1. Hematoxylin and eosin (H&E) staining: (A) group 0 at 8 wk; (B) group 100 at 2 wk; (C) group 100 at 4 wk; and (D) group 100 at 8 wk. In group 0, fibroblastic cells and no cementum-like tissue formation were observed on the dentin (d) surfaces. In group 100, new cellular cementum-like tissue (arrowheads) was demonstrated on the dentin (d) surface. Frequently resorbed dentin areas were lined by osteoblastic cells with new cementum-like tissue deposition (asterisk). Scale bar, 50 μm.

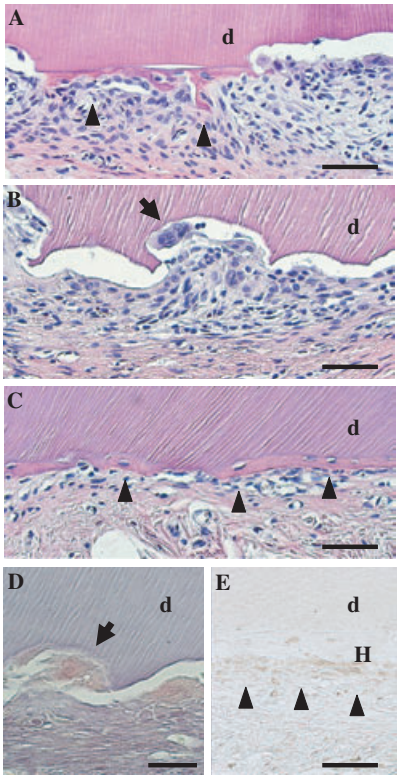

Fig. 2. (A–C) Hematoxylin and eosin (H&E) staining: (A) group 400 at 2 wk; (B) group 400 at 4 wk; and (C) group 400 at 8 wk. Dentin resorption by multinucleated cells (arrows) was shown on the dentin (d) surfaces. New cementum-like tissue (arrowheads) had formed on dentin surfaces. (D) Tartrate-resistant acid phosphatase (TRAP) staining at 4 wk. TRAP-positive cells (arrows) were observed in the resorption lacunae. (E) Osteocalcin immunolocalization at 8 wk. Osteocalcin was localized on the osteoblastic cells. Scale bar, 50 μm.

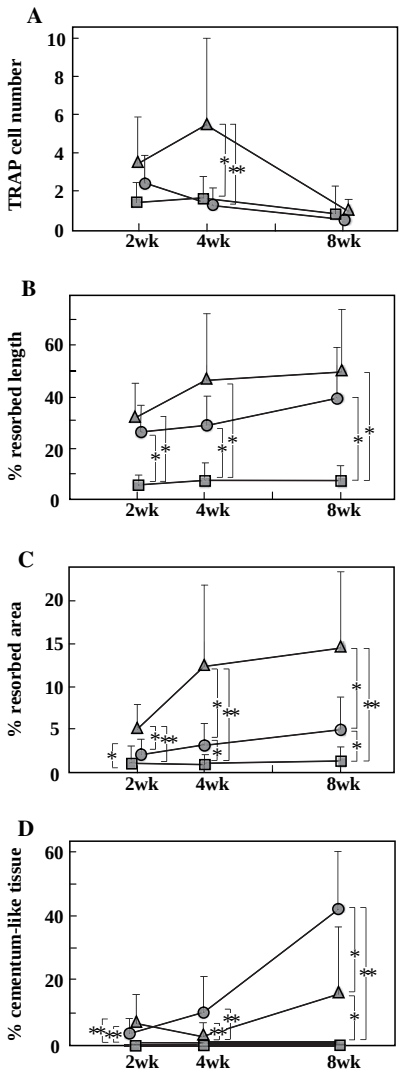

Fig. 3. Effects of bone morphogenetic protein-2 (BMP-2) application on (A) the number of tartrate-resistant acid phosphatase (TRAP)-positive cells; (B) the length of resorbed dentin surface; (C) the area of resorbed dentin; and (D) the length of new cementum-like tissue in groups 0 (■), 100 (•), and 400 (▲). The results are expressed as mean ± standard deviation (SD). $*p < 0.05$, $**p < 0.01$. Statistical differences in each group were analyzed by using the Kruskal–Wallis and Mann–Whitney *U*-test with STATVIEW®.

cementum-like tissue was poorly formed both on the nonresorbed and the resorbed dentin (Fig. 2A). In the 4-wk specimens, dentin resorption was frequently demonstrated and resorbed areas were composed of some resorption lacunae associated with TRAP-positive cells (Fig. 2B,D). At 8 wk, few osteoclastic cells, but numerous osteoblastic cells, were displayed on the dentin. Resorption lacunae were partially replaced by new cementum-like tissue (Fig. 2C). Osteocalcin was noted around the lining of osteoblastic cells at the surface of the newly induced cementum-like tissue at each experimental period (Fig. 2E).

Histomorphometric analysis

Significantly more TRAP cells were present in group 400 than in group 0 ($p < 0.05$) or group 100 ($p < 0.01$) at 4 wk (Fig. 3A).

The length of dentin resorption in groups 100 and 400 was significantly greater than in group 0 ($p < 0.01$) at all stages. Resorbed dentin surface lengths in groups 100 and 400 extended to 40% of the dentin surface, and no significant differences were found between the groups (Fig. 3B) at any of the experimental points. On the other hand, the dentin-resorbed areas in group 400 were significantly higher than those in group 0 ($p < 0.01$) or in group 100 ($p < 0.05$) at all stages (Fig. 3C).

In 8-wk specimens it was observed that new cementum-like tissue formed in 41.8% of group 100, as compared with 16.2% in group 400 ($p < 0.05$). In group 0, no cementum-like tissue formation occurred (Fig. 3D).

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 not necessary to maintain the release of BMP-2 for a long time. As a result, 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 used for future clinical trials.

Osteogenic and osteoclastic differentiations were affected by variations in the 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 4 wk were significant compared with group 100, while the data of group 400 for the dentin-resorbed area displayed consistently high levels and differed significantly compared with 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 independent of the dose of BMP-2 applied to the dentin surfaces.

Histological findings demonstrated that new cementum-like tissue formed on resorbed and/or nonresorbed dentin surfaces following the application with BMP-2. From this evidence, we speculate that two different biological mechanisms occur 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 directly by biological signal(s). On the other hand, formation of mineralized tissue was dependent on initial resorption of the denuded root surface in periodontal wound healing (16). Following the early healing of periodontal tissue, a remodeling process, with osteoclastic resorption and deposition of repair cementum, was seen at the root surface (17). Furthermore, 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 reported a dose-dependent effect of BMP-2 on the activity of calcification at the BMP-treated dentin surfaces *in vitro* (20,21). However, it is interesting to note that in the present study, more cementum-like tissue was formed in group 100 than in group 400. Some investigators reported (13,22,23) that *in vitro* bioactivities of cells were accelerated by growth factors such as BMP, platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β) at optimal concentrations, but inhibited at elevated concentrations. Thus, in the current experiment, application of an overdose concentration of BMP-2 for rats may have an inhibitory 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–cell and/or cell–extracellular matrix interactions and various biological inhibitors. Further studies are needed to explain these mechanisms.

In conclusion, this study elucidated that the dentin resorption by osteoclastic cells was facilitated by the application of a high dose of BMP-2, and cementum-like tissue formation was promoted effectively with a relatively low dose of 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.

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