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Effects of bone morphogenetic protein-6 on periodontal wound healing in a fenestration defect of rats

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Background: Bone morphogenetic proteins (BMPs) may play significant roles in bone formation. The ability of BMP-6 to promote wound healing has been chosen as the subject of this investigation. In this study, a synthetic rat BMP-6 polypeptide was applied to a periodontal fenestration defect in rats to elucidate the effects of BMP-6 on periodontal wound healing.

Material and methods: Following surgery to create a bony window on the buccal aspects of mandibular molar roots, 24 male Sprague Dawley rats were divided into four groups according to BMP application (0, 1, 3 and 10 μg, respectively). Animals were killed after 28 days and the mandible taken for histological examination. Histometric measurements were performed on sections selected from three levels (coronal, middle and apical levels; with 240 μm apart from the central) of the defect. New bone and cementum formation (including area and thickness) were analyzed and compared.

Results: In general, minimal new bone was observed on the surgically created defects in the non-BMP group, whereas a complete osseous healing occurred in all BMP-6 treated animals. New bone formation (both in area and thickness) was significantly influenced by both the dosage and the examining level, whereas new cementum formation was affected by dosage only. An increase in bone and cementum formation was noted in all three BMP groups when compared with the control group at all examined levels. Among the BMP groups, greatest new bone and cementum formation were noted in the 3 μg group. New cementum thickness increased on the cementum surfaces of the defects compared with the dentinal surfaces in all study groups.

Conclusion: An increase in new bone and cementum formation was noted after applying a synthetic BMP-6 polypeptide to a periodontal fenestration defect in rats. Therefore, we suggest that BMP-6 may play a certain role in periodontal regeneration.

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Much effort has been spent developing bioactive molecules and materials that promote periodontal wound healing, though the regulation of periodontal tissue regeneration is still under investigation (1, 2). Local application of growth factors, platelet-derived growth factor and insulin-like growth factor for instance, can

promote periodontal regeneration (3, 4). Bone morphogenetic proteins (BMPs) have attracted much interest as growth factors since Urist (5) first demonstrated that demineralized bone matrix could induce the formation of cartilage and bone in ectopic sites. Molecular cloning of genes for BMP-1, 2(2A), 3, 4(2B) and several other BMPs have been identified and cloned (6, 7). BMP-2–8 are members of the transforming growth factor-B superfamily, and can be divided further into subfamilies (6, 7). Evidence that recombinant BMPs hold promise of inducing bone formation suggests that BMPs may have enormous therapeutic potential in the management of numerous clinical conditions in which there is a requirement for new bone formation (8, 9).

Osteogenesis induced by local therapeutic administration of native or recombinant human BMPs exploits a functionally conserved process originally deployed in embryonic development (10, 11). The specific BMPs may also be used for repair and regeneration of periodontal tissues in postnatal life (1, 12). Several studies suggest that BMPs may modulate osteogenesis and cementogenesis in periodontal wounds. It has been reported that native BMPs delivered in a collagenous matrix induced cementum, periodontal ligament and alveolar bone regeneration (13). Evidence detailing substantial bone and cementum regeneration following periodontal reconstructive surgery using recombinant human BMP-2 was also demonstrated in dogs (14, 15). Recent studies in rats and dogs have successfully demonstrated enhanced periodontal regeneration following application of BMP-2 and 7 in surgically created periodontal defects (16, 17). *In vitro* experimentation has demonstrated that BMP-2 and 7 can further induce bone and cementum formation, as well as osteoblastic and chondrocytic differentiation (8, 18).

BMP-6 was originally isolated from a murine embryonic cDNA library and was named Vgr-1, based on its homology to *Xenopus* Vg-1 (19). Human and bovine homologues of Vgr-1 were subsequently isolated from bone and named BMP-6 (20). *In situ* hybridization and immunohistochemical analyses have localized Vgr-1 mRNA and protein expression in the central nervous system, in the suprabasal layer of epithelium, and, of most relevance to current studies, in hypertrophic cartilage (21–23). Notably, it is the only member of the transforming growth factor-B superfamily that has been localized to this latter region, and, as such, Vgr-1 protein could be involved in the maturation of hypertrophic cartilage and/or stimulation of osteoblastic differentiation during endochondral bone formation. Studies *in vitro* determined that BMP-6 induced the formation of more and larger bone nodules as well as increased osteocalcin secretion and that it is a 2–2.5-fold more potent inducer of osteoblast differentiation than BMP-2 or 4 (24, 25). Therefore, BMP-6 has been suspected of fulfilling an important and unique role during early stage osteoprogenitor cell differentiation (26). In addition, BMP-6 can act as an osteoinductive factor during mesenchymal differentiation (27). *In vivo* studies suggest that BMP-6 acts as an osteoinductive factor during endochondral bone formation (23, 28). However, no study to date has evaluated the effects of BMP-6 on periodontal wound healing. Thus, we applied a synthetic rat BMP-6 polypeptide to a surgically created periodontal fenestration defect in the rats to examine the effects of BMP-6 on new bone, cementum and periodontal ligament formation.

Material and methods

Experimental design

Twenty-four male Sprague Dawley rats, 9 weeks old and 300–350 g in weight, were selected and, under general anesthesia (intraperitoneal injection of 1.2 ml/kg Nembutal: Abbott Laboratories, North Chicago, IL, USA), a surgically created periodontal fenestration defect was made on the root surface of the mandibular second molar, as previously described by King *et al.* (16). In brief, an extra-oral incision was made at the base of the left

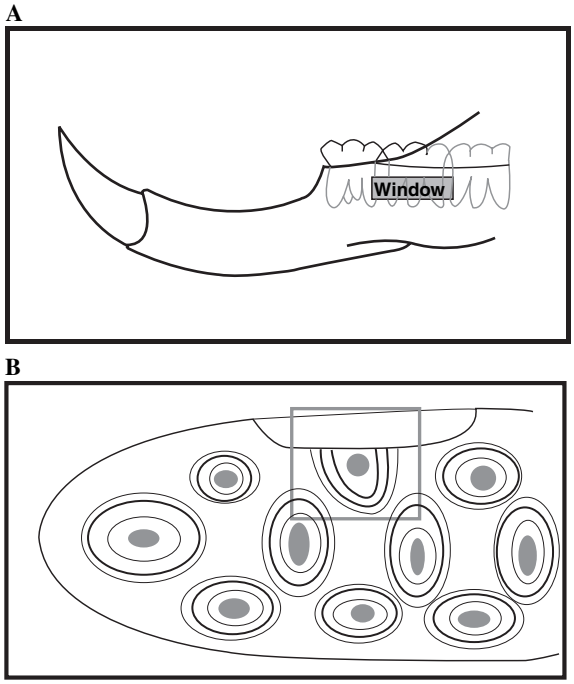

Fig. 1. Diagram representing the mandible of a rat and the surgical window defect around the buccal surface of the second molar (A), and a diagram representing the horizontal histology of the mandible containing the grid area for measurement of new bone and cementum (B).

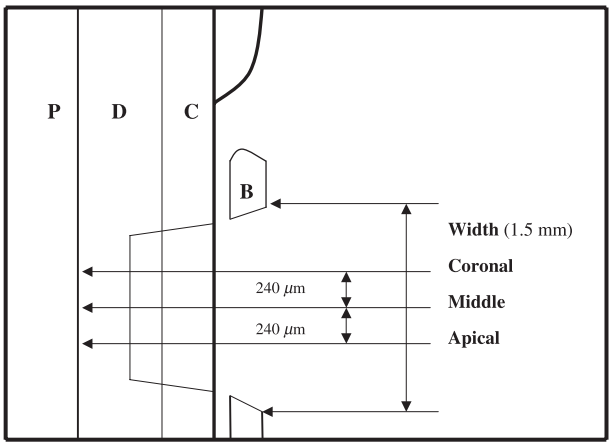

Fig. 2. Diagram representing the three histometrically examined levels (coronal, middle and apical levels) at the defect (1.5 × 3 mm in width and length). B, alveolar bone; C, cementum; D, dentine; P, pulp.

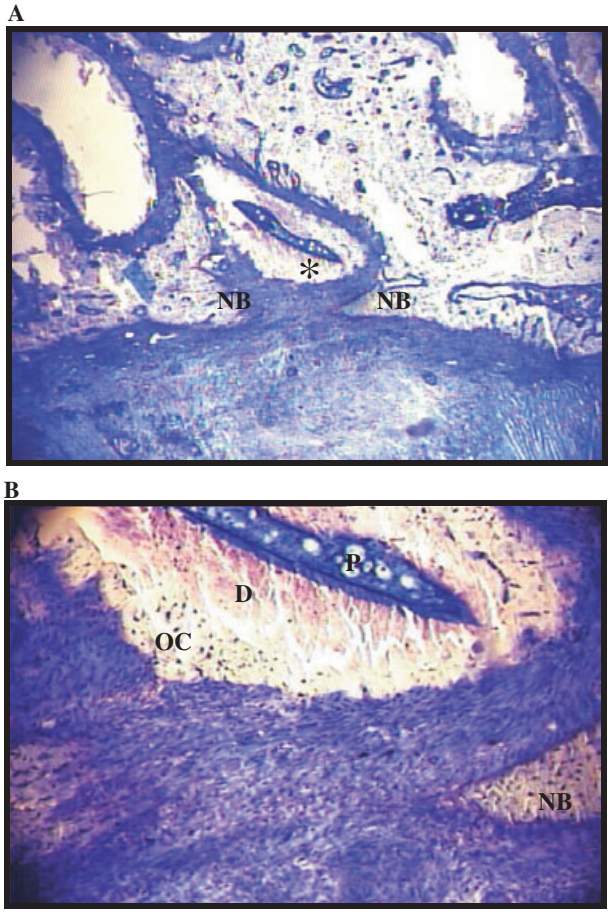

Fig. 3. A tissue section from a control rat showing the fenestration defect 28-days post-operation. (A) New bone formation on the dental alveolus beside the root (original magnification × 20). (B) Under high power magnification, a very thin layer of new cementum (if compared with that from the test animal) on the exposed dentin and cementum of the root surface (original magnification × 100). D, dentin; NB, new bone formation; OC, old cementum; P, pulp; *, buccal root of second molar; toluidine blue stain.

mandible and bone overlying the left second molar was removed with an electric drill using a size-4 round bur at slow speed under saline irrigation. The defect width was standardized to the width of the round bur (1.5 mm in diameter) and extended longitudinally (3 mm) to either side of the exposed root (Fig. 1A). The animals were then divided into four groups: a control and three BMP (1, 3 and 10 μg) groups, each including six animals. A sterile absorbable collagen sponge (1.5 × 3 × 1.5 mm) (absorbable bovine type I collagen sponge, Collacote, Zimmer Dental Inc., Carlsbad, CA, USA) impregnated with 10 μl BMP-6 (0.1, 0.3 and 1.0 μg/ μL, respectively) (Synthetic rat amino terminal sequence, Biogenesis, Poole, UK) was applied to each defect in three experimental groups, and only absorbable collagen sponge was applied to that in the control group. After suturing, the wound was left to heal for 28 days. In standardizing the surgical procedures, only one surgeon (KKH) completed the procedures for all experimental animals. At the end of experiments, all animals were killed in a 100% CO_2-filled chamber and the mandibular blocks were obtained for histological examination.

Histology preparation and histomorphometric analysis

After fixation in 70% ethanol for 24 h, the mandible tissue blocks were subsequently dehydrated in step gradients of ethanol and embedded in methyl methacrylate. Serial horizontal 8-μm sections representing the entire surgically exposed root surface from coronal to apical aspects were prepared for histological examination. Every fifth section was stained with toluidine blue. After examining the sections throughout the whole defect, three tissue sections were selected from each of the three levels, these being the middle (the central aspect of the defect), coronal and apical (with 240 μm apart from the central) levels for quantitative histological measurements (Fig. 2). The histometric measurements were performed by means of a light microscope equipped with

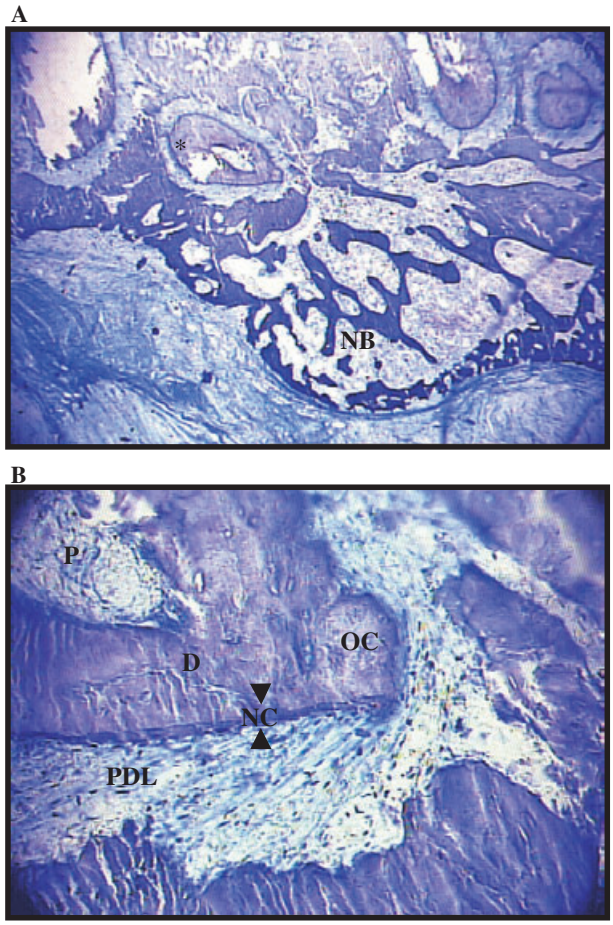

Fig. 4. A tissue section from an animal treated with bone morphogenetic protein-6 showing significant new bone formation overlying the cortical bone around the defect, especially in the distal region. (A) New bone overlying the defect contained larger and greater numbers of marrow spaces compared with the surrounding dental alveolar bone (original magnification × 20). (B) A thick layer of cementum with fibers from the root inserting into alveolar bone. No ankylosis or root resorption was observed (original magnification × 100). D, dentin; NB, new bone formation; NC, new cementum formation; OC, old cementum; P, pulp; PDL, periodontal ligament; *, buccal root of second molar; toluidine blue stain).

Table 1. Histometric analysis of new bone formation (area and thickness) following application of bone morphogenetic protein-6 in the fenestration defects in rats at 28 days

	New bone area (10^{-2} mm^2)		New bone thickness (10^{-2} mm)	
	Mean ± SE	*p*-value	Mean ± SE	*p*-value
Dosage effect				
0 μg	5.4 ± 1.7	± 0.001*	16.2 ± 10.1	± 0.001*
1 μg	28.1 ± 4.5		46.0 ± 6.8	
3 μg	46.7 ± 5.7		79.7 ± 8.5	
10 μg	29.2 ± 4.5		51.8 ± 6.8	
Level effect				
Coronal	32.8 ± 3.2	± 0.001*	55.4 ± 4.6	± 0.001*
Middle	29.0 ± 3.2		50.9 ± 4.6	
Apical	20.4 ± 2.3		38.9 ± 4.0	

Values are mean ± standard error (SE); *p*-value by repeated measures of general linear model.
*Significant difference among groups at $p < 0.05$.

a computer-assisted image analysis system (Optimas version 6.2, Media Cybernetics Inc., Silver Spring, MD, USA). The following histometric parameters of new bone and cementum formation were measured.

1 New bone area: the area of new bone formed from the cut surface of root to the level of new bone at its outer surface.
2 New bone thickness: the mean distance between the new bone outer surface and inner surface within the cut surface of root.
3 New cementum area: the new cementum formed from the cut surface of root to the level of new cementum at its outer surface.
4 New cementum thickness: the mean distance between the new cementum outer surface and inner surface within the cut surface of root. In addition, the thickness of new cementum was further evaluated at the central dentinal surface and the mesial and distal cementum surfaces of the cut root in order to determine whether any difference in new cementum formation existed among these tooth surfaces.

New connective tissue attachment and any resorption pits or ankylosis were also observed along the cut root surface. All histometric measurements were also performed by one examiner (KKH).

Data analysis

Differences among the three test and control groups, each including six animals, in the study were analyzed by repeated measures of the general linear model and Duncan's multiple comparison test for post-hoc analysis. Values were considered to be significantly different only when $p < 0.05$.

Results

Histological observation

In control groups, minimal amounts of new bone, cementum and connective attachment were observed by microscopy (Fig. 3). Residual collagen carrier was occasionally seen in a few defects. In all BMP-6 treated animals, however, a complete healing of the

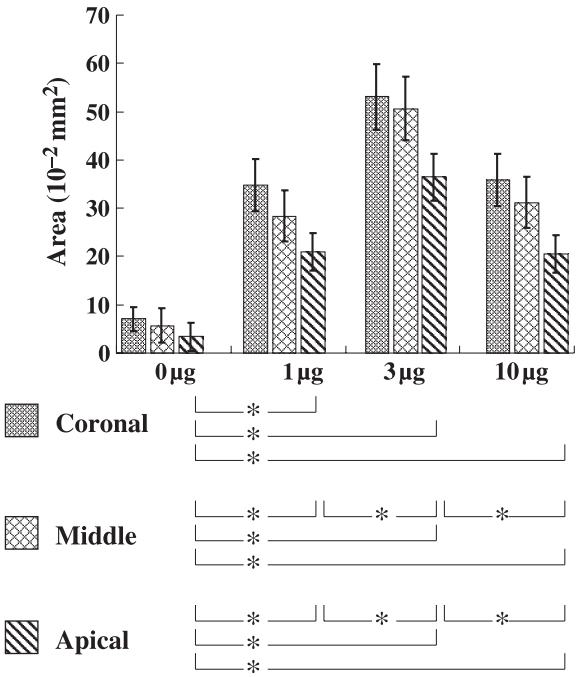

Fig. 5. Post-hoc analysis of new bone area (10^{-2} mm^2) over the defects from the test (1, 3 and 10 μg) and control (0 μg) groups at the coronal, middle and apical levels. *Statistically significant difference between each two examining groups at $p < 0.01$. Mean ± SE.

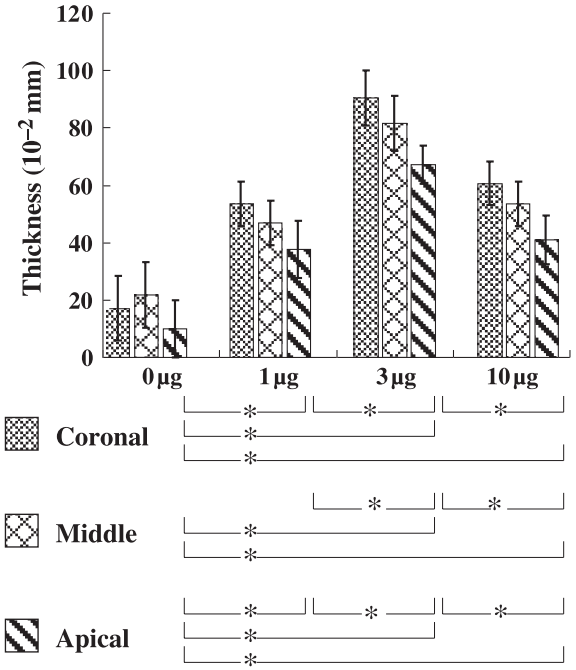

Fig. 6. Post-hoc analysis of new bone thickness (10^{-2} mm) over the defects from the test (1, 3 and 10 μg) and control (0 μg) groups at the coronal, middle and apical levels. *Statistically significant difference between each two examining groups at $p < 0.01$. Mean ± SE.

defect occurred with restoration of overlying bone, new cementum formation and functionally oriented collagen fibers (its insertion into the root surface can be identified easily by light or polarized microscopy) (Fig. 4). The new bone overlying the defect contained larger and great numbers of marrow spaces when compared to surrounding dental alveolar bone. New cementum formation was found on both old dentinal and cementum surfaces and was primarily cellular. For the 3 μg BMP group, potent increases for all periodontal wound healing hallmarks were found when compared to the other groups. No ankylosis of the bone and root was observed on any surfaces of the defect root from both the control and test animal groups. In the high dose (10 μg) group, however, an obvious resorption was observed on the root and bony surfaces adjacent to the defects when compared with the other two groups.

Histomorphometric analysis

Bone formation — Both examining factors (BMP dosages and examining levels) had significant effects on the new bone area and thickness ($p < 0.001$, Table 1). By post-hoc analysis, significant increases in new bone area and thickness were observed in all three BMP groups when compared with the control (0 μg) group, regardless of the level of section (the new bone thickness of 1 μg at the middle level being the only exception, Figs 5 and 6). Among the three BMP groups, the 3 μg group had the most pronounced effect (on both bone area and thickness) when compared with the 1 or 10 μg groups at all examined levels ($p < 0.01$), except for the new bone area at the coronal level (Figs 5 and 6).

Cementum formation — BMP dosage (but not the examining level) determined both new cementum area and thickness ($p < 0.001$ for the dosage factor in area and thickness, but $p = 0.760$ and $p = 0.207$ for the level factor in area and thickness, respectively, Table 2). By post-hoc analysis, new cementum area was increased in BMP groups when compared with the 0 μg group; however, statistically significant increases were noticed in the 3 μg group at every level and the 1 μg group at the apical level (Fig. 7). As for the bone formation previously mentioned,

Table 2. Histometric analysis of new cementum formation following application of bone morphogenetic protein-6 or carrier material (control) in fenestration defects in rats at 28 days

	New cementum area (μm^2)		New cementum thickness (μm)	
	Mean ± SE	*p*-value	Mean ± SE	*p*-value
Dosage effect				
0 μg	2.55 ± 0.90	± 0.001*	7.8 ± 2.0	± 0.001*
1 μg	4.88 ± 0.63		17.0 ± 2.0	
3 μg	8.43 ± 0.76		24.0 ± 2.0	
10 μg	4.82 ± 0.60		13.0 ± 1.0	
Level effect				
Coronal	5.36 ± 0.47	0.76	14.0 ± 1.0	0.207
Middle	5.03 ± 0.49		16.0 ± 1.0	
Apical	5.12 ± 0.40		16.0 ± 1.0	

Values are mean ± standard error (SE); *p*-value by repeated measures of general linear model.
*Significant difference among groups at $p < 0.05$.

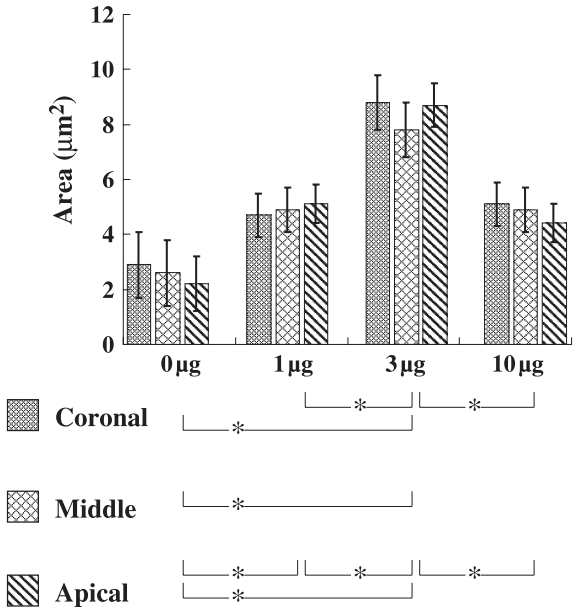

Fig. 7. Post-hoc analysis of new cementum area (10^{-2} μm^2) over the defects from the test (1, 3 and 10 μg) and control (0 μg) groups at the coronal, middle and apical levels. *Statistically significant difference between each two examining groups at $p < 0.01$. Mean ± SE.

the increases were once again more prominent in the 3 μg group when compared with the 1 μg or 10 μg groups at coronal and apical levels (Fig. 7). New cementum thickness was increased in all three BMP groups when compared with the 0 μg group, regardless of the coronal, meddle and apical levels (the statistical significance was noticed between the BMP and 0 μg groups at the all levels, except 10 μg at the middle and apical levels, Fig. 8). Again, significantly increased thickness was also found in the 3 μg group when compared with the 1 μg or 10 μg groups at all three levels (Fig. 8). In addition, decreased cementum thickness was noticed on the central dentinal surface compared with the mesial and distal cementum surfaces of the defects in all study groups (Fig. 9 and Table 3), although the BMP dosage still affected new cementum thickness on the two tooth-surfaces (Table 3).

Discussion

This study suggests that BMP-6 is one of the powerful modulators of periodontal wound healing *in vivo*. Our results showed that all histologic parameters of periodontal wound healing were improved with the single application of BMP-6, especially at the dosage of 3 μg. BMP has shown considerable promise in promoting wound healing, especially in periodontal regeneration, as it initiates the development of bony tissue by stimulating uncommitted, undifferentiated mesenchymal stem cells to convert and differentiate into a mature osteoblast phenotype (1, 29). Although the synthetic rat BMP-6 polypeptide was used in the present study, similar mechanisms of action across mammalian species have been suggested (30).

The chosen periodontal fenestration defect model is without confounding variables arising from effects of infiltrating oral bacteria and gingival epithelial ingrowth into the wound space (16). There was, however, only limited healing of the control defects at the end of experiment, even though this study utilizes an acute healing model. It seems to be a predictable and reliable model of periodontal wound healing or regeneration for the specified period (28 days). The periodontal ligament and the organic matrices of bone and dentine are composed largely of type I collagen and this matrix protein has an important role in providing a substrate for cell interaction, migration and proliferation, as well as playing a crucial role in the mineralization process (31). Collagen membrane, however, may still be a useful carrier for potent osteoinductive agents during periodontal regeneration, while at the same time utilizing both their ability to provide a scaffold for newly mineralized tissue growth and their high affinity for BMPs (32). Moreover, it was easy to mould and adapt the materials to the bone defect and root surface. Therefore, type I collagen was selected and used as the carrier of BMP-6 in this study.

Bone formation

The development of intramembranous or endochondral ossification is a complex biological process in itself, requiring intricately regulated interactions

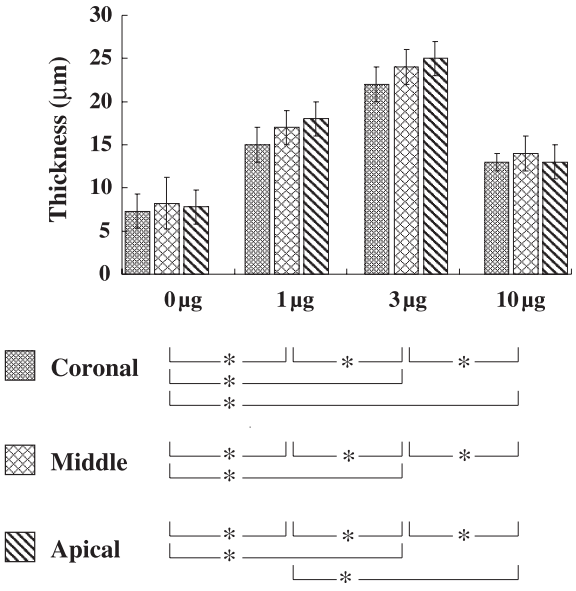

Fig. 8. Post-hoc analysis of new cementum thickness (μm) over the defects from the test (1, 3 and 10 μg) and control (0 μg) groups at the coronal, middle and apical levels. *Statistically significant difference between each two examining groups at $p < 0.01$. Mean ± SE.

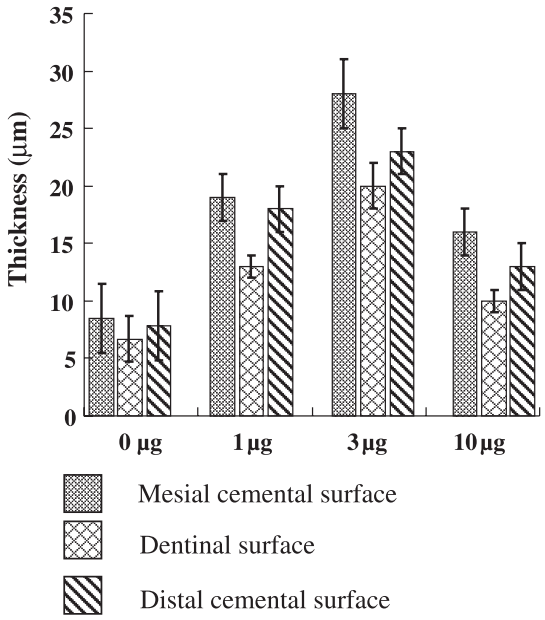

Fig. 9. New cementum thickness on the mesial cementum, central dentinal and distal cementum surfaces of the cut root from the control and three bone morphogenetic protein groups. Mean ± SE.

between cells, locally acting growth factors, mechanical loading, such as pressure and tension forces, systemic hormones and growth factors, and the matrix components in which these entities interact (33, 34). *In vivo*, BMP incorporated into two carriers (fibrous glass matrix and porous particle hydroxyapatite) produced different ossification patterns when implanted in ectopic sites (35). BMP with fibrous glass matrix showed evidence of chondrogenesis within the particles, whereas BMP with the hydroxyapatite material promoted direct or membranous ossification. Similar methods were further applied for membranous bone formation and it was reported that BMP in a type I bovine collagen membrane matrix promoted direct membranous bone formation without an endochondral precursor (36). In our present analysis, new periodontal regeneration, including the intramembranous dental alveolar bone formation, was observed after collagen-carried BMP-6 application. The other members of BMPs stimulate intramembranous bone formation during wound healing in calvarian and periodontal defects in animal studies (13, 14).

What was also noticed in our present study was that increased new bone formation centralized in both coronal and distal to the defect in the BMP groups. The reasons for this are unclear, but it is possible that BMP-6 may spread unevenly from the periodontal defect and stimulate new bone formation in tissue spaces where pooling or localized accumulation of BMP-6 occurred. Similar findings were also observed in the defect border from the study of long bone and spine in the sheep and rabbit models (37). In addition, muscle architecture may play a particular role in modulating the regeneration process.

In the present study, the density of BMP-induced new bone overlying the defect was much lower than that overlying the non-surgically involved dental alveolar bone surrounding the defect. It has been reported that BMP-induced bone formation readily produces large amounts of bone marrow (38). The concomitant development of BMP-induced bone growth with its expanded network of bone marrow ensures that both the availability of sufficient numbers of dividing stem cells and the nutritional demands for rapid new bone formation are met.

Ankylosis has been reported to be a frequent event in the submerged tooth model with BMP-2 application (14). In the study involving testing a single application of BMP-3 (osteogenin) combined with demineralized bone allograft in a submerged tooth model, increased new bone and cementum deposition around teeth after grafting was noticed; however, pinpoint

Table 3. Effects of bone morphogenetic protein (BMP)-6 dosages and different tooth surfaces on new cementum thickness

	New cementum thickness (μm)	
	Mean ± SE	*p*-value
Effect of BMP-6 dosages		
0 μg	7.7 ± 2.0	< 0.001*
1μg	17.0 ± 2.0	
3μg	24.0 ± 2.0	
10μg	13.0 ± 1.0	
Effect of tooth surfaces		
Distal cementum	18.0 ± 1.0	< 0.001*
Dentin	13.0 ± 1.0	
Mesial cementum	16.0 ± 2.0	

Values are mean ± standard error (SE); *p*-value by repeated measures of general linear model.
*Significant difference among groups at $p < 0.05$.

ankylosis was further observed (12). Other studies using recombinant human BMP-2 (39–41) or recombinant human osteogenic protein-1/BMP-7 (17) in various animal models also provide evidence of ankylosis in a variety of periodontal defects. Ankylosis of replaced incisors in a monkey model was observed at 3 weeks but subsequently reduced until it had virtually disappeared by 10 weeks (42). Similar findings that ankylosis was frequently observed at 10 days (the early stage of periodontal healing) but not at 38 days (the late stage) in rats have also been reported (16). Ankylosis has not been a complication following surgical implantation of recombinant human BMP-2 or other BMP; however, an absence of extensive bone regeneration was observed (15, 43). In the present investigation, BMP-6 was stimulating bone formation but not associated with ankylosis. In the high dose (10 μg) group, however, an obvious resorption was observed on the root and bony surfaces adjacent to the defects when compared with the other groups. A precise selection in suitable dosages was recommended due not only to the adverse effect of resorption but also to the amount of new bone and cementum formation (Figs 5–8).

Cementum formation

Our results showed that new cementum formation was increased in the test groups when compared to the control group, suggesting that BMP-6 enhanced cementum formation. BMPs may play certain roles in cementogenesis due to expression of different BMPs genes in the developing mouse tooth (44). Although the phenotypes of cementoblasts have not been fully characterized, there is clear evidence that these cells share many of the features of the osteoblast phenotype. Osteoprogenitor cells cultured on tooth root slices deposit a mineralized matrix that is morphologically and ultrastructurally similar to cementum (45, 46). The presence of exposed dentine may preferentially modulate the expression of the cementogenic phenotype on readily available cell population (pre-cementoblasts and their progenitors) from the ligament space in a periodontal defect. The expression of different phenotypes and the generation of cementum or alveolar bone by BMPs may depend on whether a common lineage of progenitor cells, residing in the periodontal ligament (47, 48), attach to exposed dentinal substrata or stay in the alveolar bony side of the periodontal ligament space.

In this study, significantly more new cementum growth was observed on existing cementum than on dentine (Fig. 9). Instrumentation on root surface has been encouraged to form new attachment; however, some reports indicated that an instrumented dentine surface presents an unsuitable surface for the formation of new cementum with attaching periodontal fibers (42, 49–51). In addition, it has been mentioned that the exposed dentin itself may interfere with new cementum formation (52). Because the activity of vanadate-resistant alkaline phosphatase was found on healing dentine surface following spontaneous marginal healing of an exposed dentine surface, Blomlof and Lindskog proposed that osteoblasts rather than cementoblasts were involved in the process of forming reparative cementum on the exposed dentine surfaces (53). They further advised that reparative cementum found on exposed dentine surfaces was the result of a transient ankylosis (42).

In our study, new cementum formation was usually cellular in nature with incorporation of Sharpey's fiber attachment. The periodontal ligament, incorporated in bone and cementum, may be oriented parallel, perpendicular or even parallel and perpendicular within the same defect. Stahl suggested that acellular cementum is more conducive for fiber attachment than cellular cementum (54). Hence, the type of cementum formation may not be a limiting factor in fiber attachment.

In conclusion, a single delivery of synthetic BMP-6 polypeptide was applied in a periodontal fenestration defect in rats to evaluate the effects of BMP-6 on periodontal wound healing. A certain type of periodontal regeneration (including new bone, cementum and periodontal ligament formation, but without ankylosis) was observed on the fenestration defect after 28 days of healing. We suggest that BMP-6 may play a certain role in periodontal regeneration. Further detailed studies are needed.

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