

Periodontal tissue regeneration with cementogenesis after application of brain-derived neurotrophic factor in 3-wall inflamed intra-bony defect

Mari Kiyota¹, Tomoyuki Iwata^{1, *}, Naohiko Hasegawa¹, Shinya Sasaki¹, Yuri Taniguchi¹, Yuta Hamamoto¹, Shinji Matsuda¹, Kazuhisa Ouhara¹, Katsuhiro Takeda^{1,2}, Tsuyoshi Fujita¹, Hidemi Kurihara¹, Hiroyuki Kawaguchi^{1,3}, Noriyoshi Mizuno¹

¹ Department of Periodontal Medicine, Hiroshima University Graduate School of Biomedical and Health Sciences, Hiroshima, 734-8553, Japan

² Department of Biological Endodontics, Hiroshima University Graduate School of Biomedical and Health Sciences, Hiroshima, 734-8553, Japan

³Department of General Dentistry, Hiroshima University hospital, Hiroshima, 734-8553, Japan

* Corresponding author: Dr. Tomoyuki Iwata, Department of Periodontal Medicine, Hiroshima University Graduate School of Biomedical and Health Sciences, Hiroshima, 734-8553, Japan
E-mail address: iwatat@hiroshima-u.ac.jp
Tel: +81-82-257-5663

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Abstract

Objective: The purpose of this study is to investigate regenerative process by immunohistochemical analysis and evaluate periodontal tissue regeneration following a topical application of BDNF to inflamed 3-wall intra-bony defects.

Background: Brain-derived neurotrophic factor (BDNF) plays a role in the survival and differentiation of central and peripheral neurons. BDNF can regulate the functions of non-neural cells, osteoblasts, periodontal ligament cells, endothelial cells, as well as neural cells. Our previous study showed that a topical application of BDNF enhances periodontal tissue regeneration in experimental periodontal defects of dog and that BDNF stimulates the expression of bone (cementum) related proteins and proliferation of human periodontal ligament cells.

Methods: Six weeks after extraction of mandibular first and third premolars, 3-wall intra-bony defects were created in mandibular second and fourth premolars of beagle dogs. Impression material was placed in all of the artificial defects to induce inflammation. Two weeks after the first operation, BDNF (25 and 50 µg/ml) immersed into atelocollagen sponge was applied to the defects. As a control, only atelocollagen sponge immersed in saline was applied. Two and four weeks after the BDNF application, morphometric analysis was performed. Localizations of osteopontin (OPN) and proliferating cell nuclear antigen (PCNA)-positive cells were evaluated by immunohistochemical analysis.

Results: Two weeks after application of BDNF, periodontal tissue was partially regenerated. Immunohistochemical analyses revealed that cells on the denuded root surface were positive with OPN and PCNA. PCNA positive cells

were also detected in the soft connective tissue of regenerating periodontal tissue. Four weeks after application of BDNF, the periodontal defects were regenerated with cementum, periodontal ligament, and alveolar bone. Along the root surface, abundant OPN positive cells were observed. Morphometric analyses revealed that percentage of new cementum length and percentage of new bone area of experimental groups were higher than control group and dose-dependently increased.

Conclusion: These findings suggest that BDNF could induce cementum regeneration in early regenerative phase by stimulating proliferation of periodontal ligament cells and differentiation into periodontal tissue cells, resulting in enhancement of periodontal tissue regeneration in inflamed 3-wall intra-bony defects.

1. Introduction

Principal purpose of periodontal regenerative therapy is functional regeneration of alveolar bone, cementum and periodontal ligament. Previous studies have shown that the conventional periodontal regenerative therapies, guided tissue regeneration (GTR) and application of enamel matrix derivative, successfully regenerate periodontal tissue^{1,2}. Production of various growth factors has facilitated in vitro and in vivo studies to determine the efficacy of growth factors in periodontal tissue regeneration. Several growth factors, including platelet-derived growth factor (PDGF) combined with insulin-like growth factor-I (IGF-I)³, basic fibroblast growth factor (bFGF)⁴, bone morphogenetic protein-2 (BMP-2)⁵ and osteogenic protein-1 (OP-1)⁶, have shown to regulate migration, attachment, proliferation, and/or differentiation of periodontal ligament cells into hard tissue forming cells and enhance periodontal tissue regeneration in vivo.

Brain-derived neurotrophic factor (BDNF) is the second member of the neurotrophin family. The neurotrophin family in mammals consists of four members: nerve growth factor (NGF), BDNF, neurotrophin-3 (NT-3), and NT-4/5. Neurotrophins are essential for neuron differentiation, function, and survival in peripheral and central nerve systems^{7, 8}. BDNF exerts its biologic functions by binding to high-affinity receptor trkB (tyrosine protein kinase receptor for BDNF) and low affinity receptor, p75^{8, 9}. Furthermore, BDNF is expressed in various non-neural tissue: tooth germ¹⁰, bone¹¹, cartilage¹¹, heart¹², spleen¹³, placenta¹⁴, prostate¹⁴ and kidney¹⁴. Vascular endothelial cells¹⁵, osteoblastic cells¹⁶, and immune cells^{17, 18} express BDNF. Thus, BDNF can regulate the functions of nonneural cells as well as neural cells.

Our previous study showed that cultured human periodontal ligament (HPL) cells have

been found to produce BDNF and TrkB¹⁹. It is also shown that BDNF stimulates the expression of bone (cementum) related proteins, the proliferation of human periodontal ligament cells²⁰⁻²² and that a topical application of BDNF to experimental class III furcation defects enhanced periodontal tissue regeneration in dogs²³⁻²⁶. Therefore, regulating the function of periodontal ligament cells by BDNF may enhance complete periodontal tissue regeneration.

The results of previous study using experimental class III periodontal defects demonstrated that comparatively large defects could be successfully regenerated by the BDNF application²³⁻²⁶. However, experimental defects used in previous study were surgically created acute defect. Since, in acute defects, some degree of spontaneous repair must be taken into consideration, we needed to evaluate their regenerative possibility in chronic periodontal defect. Furthermore, class III periodontal defects are too large size defects to observe detailed regenerative process with some accuracy, because of the sensitivity of the interpretation of regenerative techniques.

To investigate a more detailed regenerative process, the regeneration process in a much smaller size of the bone defect should be observed. Moreover, in terms of actual clinical application, regenerative therapy using signaling molecules is applied realistically for use in 2- and 3-wall intra-bony defects. Therefore, we selected an inflamed 3-wall intra-bony defect. Probably due to such a smaller size of the defect, we can discuss some stages of periodontal tissue regeneration and investigate further information.

In this study, we evaluated periodontal tissue regeneration after a topical application of BDNF to inflamed 3-wall intra-bony defects in beagle dogs, including new bone and new cementum formation, by Morphometric analyses. Subsequently, we investigated localizations of osteopontin (OPN) and proliferating cell nuclear antigen (PCNA)-

positive cells immunohistochemically to elucidate detailed regenerative process.

2. Materials and Methods

2.1. Animals

After approval from the Committee of Research Facilities for Laboratory Animal Science, Hiroshima University School of Medicine, was obtained. 15 female beagle dogs weighing 10 -14 kg and with ages of 12 - 20 months were used in this study.

2.2. Surgical protocol

All surgical procedures were performed under sodium pentobarbital anesthesia (40mg/kg) and local infiltrated anesthesia with 2% lidocaine with 1:80000 noradrenaline. Periodontal tissues around mandibular second and fourth premolars (P2 and P4) in each dog were selected for experimentation. Mandibular first and third premolars (P1, P3) were extracted, and 6 weeks after the extraction, creation of 3-wall intra-bony defects were performed. After sulcular incisions, mucoperiosteal flaps were raised and 3x3x4mm 3-wall intra-bony defects were created at the mesial and distal aspects of P2 and mesial aspect of P4 (fig.1). Supporting bone and periodontal ligament were removed with steel burs and exposed cementum of the teeth was removed with Gracey curettes. Reference notches were placed on each root surfaces to indicate the top and the base of the defect. Impression material was, then, placed in all of the artificial defects to induce inflammatory responses against the foreign body.

Two weeks after the operation, we raised the flap to expose the defects, removed impression material and granulation tissue and planed root surfaces. BDNF (R&D

Systems, Minneapolis, MN) was diluted with normal saline (Otsuka Pharmaceutical, Tokyo, Japan) to 25 and 50µg/ml. The BDNF was immersed into atelocollagen sponge (8mm diameterx3mm; Terumo, Tokyo, Japan) and applied to the defects. As a control, Atelocollagen sponge immersed in saline was applied. The flaps were repositioned with 4-0 silk sutures.

2.3.Tissue preparation

During the postoperative observation periods, good oral hygiene was maintained by brushing and swabbing with 0.2% povidone iodine (Meiji-seika Co., LTD., Tokyo, Japan).

One, two, and four weeks after the application, anesthetized animals were sacrificed by vascular perfusion with 4% paraformaldehyde in sodium cacodylate buffer containing 0.05% calcium chloride (pH 7.3). The mandibles were dissected and decalcified with 10% EDTA for 2 weeks. They were dehydrated through graded ethanol, cleared with xylene and embedded in paraffin. Serial sections (5µm) were cut in the mesial-distal plane throughout the buccal-lingual extension of the tooth. The sections were stained with hematoxylin and eosin (HE) and observed using a light microscope.

2.4. Morphometric analysis

Nine premolars had not been available because of technical failure, 21 teeth from five dogs were used for morphometric analysis of three groups (control and BDNF 25 and 50µg/ml). 21 sections from 7 teeth (3 sections /tooth) were given to each group. The technical failure was happened in making bone defect model and the major failure was being exposed pulp when drilling the alveolar bone.

Other failures were occurred during slicing and section preparation, resulting in artificial

scratches on the sections.

For histometric analysis, sections of the most central area of the defect and buccal and lingual area 100µm apart from the central were used. The length of newly formed cementum, the height and the area of newly formed bone were measured with NIH image software on digitalized photomicrographs. New cementum formation (NCF) was represented as the percentage of length of new cementum to the total root surface length from notch to notch. The height of newly formed bone (NBH) was represented as the percentage of length of new bone-height to the total root surface length from notch to notch. The area of newly formed bone on each specimen (NBA) was calculated as the percentage of the area surrounded with reference notches on the root surfaces and bone defect (Fig.2). Because the periodontal ligament space is presented in normal periodontal tissue, percentage of bone area in normal specimens was also calculated. All data were statistically analyzed using the Mann-Whitney U test.

2.5. Azan staining

Some sections were stained according to the AZAN method to observe collagen fibers as previously described ^{25, 27}. Briefly, deparaffined and hydrated sections were immersed in azocarmine G solution for 30 min at 56-60°C, rinsed with aniline alcohol solution and 1% acetic acid in ethanol, soaked in 5.0% phosphotungstic acid aqueous solution for 60 min at room temperature, and dipped in aniline blue-orange G solution for 30 min at room temperature.

2.6. Immunohistochemical procedures

Immunohistochemical procedures for OPN ^{23, 24, 28} and PCNA ^{23, 29} were performed as

previously described. Briefly, rabbit polyclonal anti-human OPN antibody (LF123, courtesy of L.W. Fisher, Craniofacial and skeletal disease Branch, National Institute of Health, Bethesda, BD), mouse monoclonal anti-proliferating cell nuclear antigen (PCNA, DAKO Corporation, Carpinteria, CA) were used as primary antibodies. Sections were deparaffined with xylene, rehydrated through a descending ethanol series and washed in distilled water. Endogenous peroxidase was blocked by incubating the sections with 3% hydrogen peroxide. After washing sections with Tris buffered saline (TBS, pH 7.2), they were treated with 0.1% bovine serum albumin (Sigma, St. Louis, MO) to prevent nonspecific binding. Then the following incubations were performed. The primary antibodies were diluted in DAKO antibody diluent (DAKO Corporation) and incubated for approximately 1 h at room temperature. The primary antibodies dilutions were: OPN (1:400), PCNA (1:200). After incubation with the primary antibodies, sections were rinsed with TBS and incubated with ENVISION (DAKO Corporation) for 30 min in a moist chamber. These slides were rinsed in TBS. Antibody complexes were visualized with 3,3'-diaminobenzidine (DAB) substrate, washed in distilled water and counterstained with hematoxylin. As controls, some sections were treated in the same way with the exception of incubation with the primary antibodies. The differential labeling patterns obtained with various antibodies also served as internal controls.

All OPN-positive cells or PCNA-positive cells around the root surface in each section were counted, and these values represent the average of OPN-expressing cells or PCNA-expressing cells in the section. The intensity of OPN or PCNA expression around the root surface in each section was measured by image analysis using Image J software (National Institutes of Health, Bethesda, MD, USA), and these values represent the percentage of intensity relative to the control.

2.7. cells

All human bone marrow-derived MSCs (hBM-MSCs) derived from ilium bone marrow were provided by RIKEN Bio-resource Center (Tsukuba, Ibaragi, Japan) and all human periodontal ligament cells (HPL cells) were purchased from Lonza (Walkersville, MD, USA), and cultured in 100-mm plates (Corning, Corning, NY, USA) in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS; Hyclone®; GE Healthcare Life Sciences, Pittsburgh, PA, USA), 100 units/mL penicillin (Invitrogen, Carlsbad, CA, USA) and 100 µg/mL streptomycin (Invitrogen) with or without BDNF (50 µg/ml; R&D Systems) at 37 °C in a 5% CO₂.

2.8. Real-time quantitative RT-PCR and mRNA array

Total RNA was isolated from cells with RNAiso Plus (Takara Bio Inc., Shiga, Japan). cDNA was synthesized using ReverTra Ace® (TOYOBO, Osaka, Japan) with oligo (dT) primer for real-time quantitative RT-PCR analysis was conducted using TaqMan® probes and primers for cementum protein 1 (CEMP1; Assay number: Hs03004478_s1), protein-tyrosine phosphatase-like member-A (PTPLA; Assay number: Hs00171965_m1), F-spondin (SPON; Assay number: Hs01120488_m1), runt-related transcription factor 2 (RUNX2; Hs00231692_m1), and β-actin (assay no. 4310881E) with ABI StepOnePlus (Applied Biosystems, Foster City, CA, USA).

2.9. Statistical analysis

Experimental results are presented as the mean ± standard deviation. The Mann–

Whitney U test (Fig.6) or Students t-test (Figs.S1 and S2) was performed to detect statistically significant differences in the morphometrical values or the fold-induction of mRNA expression levels respectively. Results with p-values < 0.05 were considered significant.

3. Results

3.1. Histological analysis

One week after the application, periodontal tissue regeneration was not observed in both of the BDNF groups and control group, and infiltration of inflammatory cells was observed in the connective tissue of the defects in the control group (Fig.3A and 3B). Atelocollagen remained in the connective tissue of the defect (Fig.3C). Infiltration of inflammatory cells was observed in the connective tissue of the defect in control groups (Fig.3D) and, moreover, soft tissue was not contacted to dentin surface compared to the BDNF group (Fig. 3D).

Immunohistochemical study revealed that OPN expression in the BDNF group was localized on the denuded root surface (Fig.3E) and that of the control group was expressed less than that of the BDNF group and scattered throughout the soft tissue (Fig.3F). OPN-expressing cells around the denuded root surface in the BDNF groups are significantly more than that in the control group (Fig. S1A) and the expression intensities are also greater than that in the control group (Fig. S1B). Furthermore, cells in the connective tissue and on the denuded root surface were positive with PCNA and the expression in the BDNF group (Fig.3G) was more than that of the control group (Fig. 3H). Focused on

cells around these denuded root surfaces, PCNA-positive cells in the BDNF groups were also more than that in the control group but not significant (Fig. S1C), but the expression intensities were enhanced more than that in the control group (Fig. S1D).

Two weeks after the application, new bone formation in BDNF groups was limited in the bottom area of the defect (Fig.4A). Bone regeneration in control group was also limited and epithelial cells invaded into root surface (Fig.4B). In the BDNF groups, many of cells were observed in the connective tissue and around the new bone (Fig.4C). On the other hand, soft tissue of the coronal side in the control group was still not contact to root surface and there is less new bone than that of BDNF group (Fig.4D). In the BDNF groups, cells on the denuded root surface were immunoreactive for OPN (Fig.4E) and the number of OPN-expressing cells was significantly more than that of the control group (Fig. S1A). The OPN expression was more intense than that of the control group (Fig.4F) and the difference was significant (Fig. S1B). At the same area on the root, PCNA positive cells were existed in regenerating soft connective tissue facing new cementum and near new bone in addition to the central part of gingival connective tissue (Fig.4G) and insertion of new collagen fibers into new cementum was observed by AZAN staining (Fig.4H).

Four weeks after application, in the BDNF group, significant amount of new bone and adequate width of periodontal ligament were observed (Fig.5A). In control group, periodontal tissue regeneration was insufficient and cementum regeneration was limited (Fig.5B). The denuded root surface was almost completely covered with new cementum and regenerated periodontal ligament separated the new bone from cementum (Fig.5C). As to the control group, soft tissue was still not separated from root surface and remarkable new bone was not detected (Fig.5D). OPN positive cells were existed in the whole area of the defect. Along the root surface, abundant OPN positive cells were

observed (Fig.5E). Contrary, OPN expression intensity along with the root surface was decreased to a low level that hardly be detected despite the higher OPN expression area observed in the far area from detached gingival connective tissue in the control group (Fig.5F). The number of OPN-positive cells around the root surface in the BDNF group was still sustained significantly higher than that in the control group (Fig. S1A) but the expression intensity in the BDNF group was attenuated and became significantly weaker than that of the control group (Fig. S1B). Immunohistochemical study showed that cells on the root surface showed weak staining with PCNA (Fig.5G). Moreover, Azan staining showed new collagen fibers inserted into new cementum and bone (Fig.5H).

3.2. Morphometric analysis

The length of newly formed cementum, the height and the area of newly formed bone were measured according to the schema on Fig.2.

The percentages of new cementum formation in 25 and 50 μ g/ml groups were $84.8\% \pm 4.5\%$ and $84.7\% \pm 7.9\%$, respectively, compared to $43.7\% \pm 10.9\%$ in the control group (Fig.6A). The percentages of new bone length in 25 and 50 μ g/ml groups were $58.7\% \pm 5.5\%$ and $75.2\% \pm 8.1\%$, respectively, compared to $31.2\% \pm 12.0\%$ in the control group (Fig. 6B). The percentages of new bone area in 25 and 50 μ g/ml groups were $67.3\% \pm 18.2\%$ and $82.3\% \pm 7.6\%$, respectively, compared to $22.2\% \pm 11.9\%$ in the control group (Fig. 6C).

3.3. Genetic analysis

At 24 hours after application of BDNF for MSC and HPL cells culture, RUNX2, one of the osteogenesis-related genes, was induced (Fig. S2).

For cementogenesis-related genes in MSC culture, CEMP1 and SPON mRNA were significantly induced by BDNF application except for PTPLA1 mRNA (Fig.S2A).

Furthermore, HPL cells culture also resulted in upregulation of mRNA expression for Runx2, CEMP1, and SPON, paralleling the trends observed in MSC cultures (Fig. S2B). However, PTPLA1 mRNA expression was significantly suppressed in HPL cell cultures although it remained unaffected in MSC cultures in response to BDNF.

4. Discussion

To evaluate possibility of enhancing tissue regeneration by a topical application of BDNF to inflamed defects, we caused experimental inflammation after creation of periodontal tissue defect. The results of present investigation demonstrated that the defects were almost regenerated with cementum, periodontal ligament, and alveolar bone, four weeks after the application of BDNF. Epithelial downgrowth, ankylosis, or root resorption was not shown in experimental groups. Present histological analysis showed that a topical application of BDNF induced significant periodontal tissue regeneration in inflamed 3-wall intra-bony defect.

In this study, we measured new cementum formation and new bone formation. Four weeks after the application, significant periodontal tissue regeneration was observed in the experimental group compared with the control group. This result was in consistent with our previous report ²³⁻²⁶. Concentration of BDNF is an important factor for tissue regeneration. Based on the previous study, we examined two concentrations of BDNF (25 and 50µg/ml). Among concentrations used in this study, NBA, NBH and NCF were increased in a dose dependent manner. This result indicate same tendency with previous study ²³⁻²⁶. Higher concentration (50µg/ml) is considered to be effective for

periodontal tissue regeneration. However, additional studies with various concentrations in various conditions will extend our knowledge on viable tissue engineering.

It is generally accepted that new cementum formation and restoration of soft connective tissue attachment to the cementum are crucial to establish the functional periodontal tissue regeneration ^{1, 2, 30, 31}. The present results showed that OPN expression was localized on the denuded root surface one week after the application and that new cementum with extrinsic fibers were formed along the denuded root surface four weeks after the application. It is noteworthy that epithelial down growth were not observed in the BDNF group. Epithelial down growth are not favorable repair phenomena, as they interfere with the completion of periodontal tissue regeneration. BDNF induce cementogenesis ^{20, 32} and OPN expression in periodontal tissue ^{20, 22}, moreover, BDNF regulates cell growth of gingival epithelial cells ^{33, 34}. Therefore, newly formed cementum covering with denuded root surface might prevent epithelial downgrowth in early regenerative phase. It has been hypothesized that the type of tissue predominates in the wound healing determines whether the response is one of the repair or regeneration ^{1, 35}. Therefore, inducing cementum regeneration in early regenerative phase is considered to be critical for functional periodontal tissue regeneration.

Previous study showed that BDNF stimulates DNA synthesis in mouse periodontal ligament cells ^{36, 37}. Other studies reported BDNF stimulated proliferation of human periodontal ligament cells ^{19, 25}. In this study, we investigated proliferation of cells in the defect in regenerative process immunohistochemically. One week and two weeks after the application, in the connective tissue near the denuded root surface, abundant PCNA positive cells were observed in addition to connective tissue. These results

demonstrated that BDNF could also stimulate proliferation of periodontal ligament cells of dog in vivo study. PCNA-positive cells ubiquitously exist in gingival connective tissue as well as around the root surface. These cells are gingival fibroblasts, therefore, BDNF can also stimulate gingival fibroblasts and affect cell proliferation of gingival fibroblasts. However, gingival fibroblasts are not multipotency but periodontal ligament cells have multipotency and can differentiate into periodontal cells³⁸. Therefore, BDNF can relatively enhance the activity of differentiation by promoting the proliferation of periodontal ligament cells around the root surface. However, four weeks after the application, less PCNA positive cells were observed on the denuded root surface than after one week or two weeks. Based on these findings, it is indicated that BDNF could stimulate migration of periodontal ligament cells to the denuded root surface and proliferation of resident cells in the early regenerative phase. In the late regenerative phase, cells in the defect may have almost differentiated into periodontal ligament composing cells.

In the histological determination, most of control groups show the detachment of gingival epithelium until 4 weeks. The loss of the connective tissue attachment is caused by sustainable inflammation³⁹. Gingival connective tissue at the bottom of bone defect in the control group remained more inflammatory cell infiltration than that of BDNF groups (Figs. 3, 4, and 5). Moreover, the recovery of gingival epithelial attachment can come from the anti-inflammatory effect of BDNF⁴⁰. Furthermore, BDNF positively regulates periodontal cell function in periodontal tissue²⁵. Therefore, BDNF can accelerate the recovery of adhesion of gingival connective tissue and root surface, and it enhance periodontal regeneration.

Furthermore, BDNF application for periodontal regenerative therapy should be required

to a suitable carrier as BDNF has a short half-life and can be degraded easily ⁴¹. Based on the report that a collagen sponge can release rhBMP2 for a week or more ⁴², a collagen sponge can also sustainably release BDNF for about a week and a collagen sponge as a carrier for BDNF in our previous study ²⁵ and fine periodontal regeneration was confirmed by BDNF/collagen sponge. Therefore, collagen sponge is suitable as a carrier for BDNF.

OPN is non-collagenous protein predominantly accumulating at tissue interface in bone and teeth and plays important roles in cementogenesis and cementum regeneration ⁴³⁻⁴⁶. Moreover, accumulation of OPN is a primary event during the formation of regenerative cementum onto denuded root surfaces ^{47,48}. We have investigated localization of OPN in periodontal tissue regeneration to reveal regenerative process. Results of two and four weeks after the application showed that OPN positive cells were observed on the denuded root surface. Our previous in vitro study showed that BDNF elevated the expression of ALPase and osteocalcin mRNAs and increased the synthesis of OPN, BMP-2, and type I collagen DNA in HPL cells ²⁵. These findings suggested that BDNF could stimulate differentiation of resident cells into periodontal tissue composing cells; osteoblast, cementoblast, or fibroblast, following the proliferation of resident cells. Furthermore, it is assumed that differentiation into cementoblast might be induced prior to differentiation into osteoblast.

BDNF is involved in angiogenic action of endothelial cells ^{25,49}. In our previous study, we found that BDNF stimulated angiogenesis, mRNA expression of vascular endothelial growth factor-B and tenascin-X, and proliferation of human endothelial cells ²⁵. In vitro effect of BDNF in endothelial cell support the possibility that periodontal tissue regeneration enhanced by BDNF might be result from stimulating endothelial cells as

well as periodontal ligament cells.

The biological effects of BDNF are mediated through TrkB, a high-affinity transmembrane receptor, and p75, the low-affinity receptor which is a member of tumor necrosis factor receptor superfamily^{22, 50}. Our previous study showed that human periodontal ligament cells expressed TrkB mRNA¹⁹. In our immunohistochemical study of dog, TrkB localized in osteoblast, cementoblast, and fibroblast of healthy and regenerating periodontal tissue in both of the BDNF group and control group (unpublished data). The mechanisms by which the trophic effects of BDNF mediated via TrkB and p75 are still unconfirmed. Further immunohistochemical studies will be required to clarify significance of TrkB and p75 in periodontal tissue regeneration.

Other growth factors, including PDGF, IGF-I, FGF-2, BMP-2, and OP-1 have investigated therapeutic potential for periodontal tissue regeneration^{3-6, 51}. PDGF and IGF-1 can stimulate the proliferation and chemotaxis of periodontal ligament cells⁵². PDGF also stimulate collagen synthesis of periodontal ligament cells⁵³. BMP-2 has the potential to regenerate cementum and bone⁵⁴. However, BMP-2 elicits ankylosis as well as the new formation of cementum and bone^{55, 56}. The application of FGF-2, as well as BDNF, results in enhancement of periodontal tissue regeneration without epithelial downgrowth and ankylosis^{4, 57}. FGF stimulate the proliferation of human gingival epithelial cells⁵⁸ and decrease the type I collagen synthesis⁵⁹. In contrast, BDNF did not affect the proliferation of human gingival epithelial cells and enhanced the type I collagen synthesis^{25, 34}. Various growth factors enhanced periodontal tissue regeneration by different mode of stimulation to periodontal ligament cells. Further investigation will be required to elucidate the precise mechanism of periodontal tissue

regeneration by each growth factor.

Moreover, BDNF, as well as some other cytokines applied for periodontal regeneration therapy, regulates the cementogenesis of undifferentiating multiple progenitor cells, for example, mesenchymal stem cells. These cytokines characteristically enhance cementoblast-related or periodontal ligament cell-related genes and microRNAs^{60, 61}. Therefore, the function of BDNF and the other cytokines can be distinguished in the periodontal regenerative process and effect, and these cytokines can creatively be applied based on each morphology of bone defects.

5. conclusion

Our result indicates that BDNF could induce cementum regeneration in the early regenerative phase by stimulating the proliferation of periodontal ligament cells and differentiation into periodontal tissue cells, resulting in enhancement of periodontal tissue regeneration in inflamed 3-wall intra-bony defect.

Further studies for understanding regenerative process and more effective condition to apply BDNF can yield highly beneficial clinical applications for regenerating periodontal tissue.

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Author Contributions: Dr. Mari Kiyota designed and performed the experiments; collected, analyzed, and interpreted the data; and wrote and edited the manuscript; Drs. Tomoyuki Iwata and Naohiko Hasegawa wrote and edited the manuscript; Drs. Shinya Sasaki, Yuri Taniguchi, Yuta Hamamoto, Shinji Matsuda, Kazuhisa Ouhara, Katsuhiro Takeda, and Tsuyoshi Fujita performed the experiments. Drs. Hidemi Kurihara, Hiroyuki Kawaguchi, and Noriyoshi Mizuno supervised all aspects of the study as the senior investigator and director of the laboratory.

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Figure Legends

Fig. 1. Presurgical view of the mandibular premolar area

Before bone defect preparation (A); Preparation of 3-wall intrabony defect (3x3x4mm) (B); Two weeks after bone defect preparation (C); After the application of BDNF immersed into atelocollagen sponge (D).

Fig. 2. Schematic drawing of morphometric analysis of periodontal tissue regeneration

percentages of new cementum formation, new bone height and new bone area.

Fig. 3. One week after the application of BDNF.

A, B) Periodontal tissue regeneration was not observed in both of the BDNF group and control group. B) Infiltration of inflammatory cells was observed in the connective tissue of the defects in the control group (asterisk). C, E) Higher magnification of square area of X. D, F) Higher magnification of square area of Y. E) OPN expression was localized on the denuded root surface in the BDNF groups. F) OPN expression was expressed the gingival connective tissue in the control groups.

Arrowheads indicate the apical border of the denuded root surface.; line: border of the defect; d: root dentin

A, B, C, D: HE staining; E, F: immunohistochemical staining of OPN; G, H: immunohistochemical staining of PCNA; scale bars: A, B=200 μ m; C, D, E, F, G, H:20 μ m.

Fig. 4. Two weeks after the application.

A) New bone formation in BDNF group was limited in the bottom area of the defect. B) New bone formation in control group was also limited and epithelial cells (arrow) invaded into root surface. C, E, G, H) Higher magnification of square area of X. D, F) Higher magnification of square area of Y. E) In serial sections, cells on the denuded root surface were immunoreactive for OPN in the BDNF group. F) OPN expression was evenly expressed the gingival connective tissue in the control groups. G) PCNA positive cells were existed in regenerating soft connective tissue facing new cementum and near new bone. H) Insertion of new collagen fibers into new cementum was observed by AZAN staining.

Arrowheads indicate the apical border of the denuded root surface.; line: border of the defect; d: root dentin; b: new bone

A, B, C, D: HE staining; E, F: immunohistochemical staining of OPN; G: immunohistochemical staining of PCNA; H: Azan staining; scale bars: A, B=200 μ m; C, D, E, F, G, H:20 μ m.

Fig. 5. Four weeks after the application.

A) Significant amount of new bone and adequate width of periodontal ligament were observed. B) In control group, periodontal tissue regeneration was insufficient and epithelial cells invaded into root surface (arrow). Infiltration of inflammatory cells was observed in the connective tissue of the defect (asterisk). And no cementum regeneration was observed in the area. C, E) Higher magnification of square area of X. D, F) Higher magnification of square area of Y. E) Along the root surface, abundant OPN positive cells were observed in the BDNF group. F) OPN expression was decreased in the control group. G) Cells on top of the new bone surface showed weak staining with

PCNA. H) Insertion of new collagen fibers into new cementum was observed by AZAN staining.

Arrowheads indicate the apical border of the denuded root surface.; line: border of the defect; d: root dentin; b: new bone

A, B, C, D: HE staining; E, F: immunohistochemical staining of OPN; G: immunohistochemical staining of PCNA; H: Azan staining; scale bars: A, B=200 μ m; C, D, E, F, G, H:20 μ m.

Fig. 6. Histometrical analysis (4 weeks after application)

Morphometric analysis of three groups (control and BDNF 25 and 50 μ g/ml) are performed. 21 sections from 7 teeth (3 sections /tooth) were given to each group.

The length of newly formed cementum, the height and the area of newly formed bone were measured. A. New cementum formation (NCF) was represented as the percentage of length of new cementum to the total root surface length from notch to notch. B. The height of newly formed bone (NBH) was represented as the percentage of length of new bone-height to the total root surface length from notch to notch. C. The area of newly formed bone on each specimen (NBA) was calculated as the percentage of the area surrounded with reference notches on the root surfaces and bone defect. The values represent the percentage of them (mean \pm standard deviation; * $p < 0.01$; Mann-Whitney U test.; $n = 7$).

Fig. S1. Immunohistochemical analysis.

A. Cell numbers of OPN-expressing cells around the root surface. Each OPN-expressing cell was counted at 1w, 2w, and 4w. B. Intensity of OPN expression around the root

surface. Each intensity was measured at 1w, 2w, and 4w. C. Cell numbers of PCNA-expressing cells around the root surface. Each PCNA-expressing cell was counted at 1w. D. Intensity of PCNA expression around the root surface. Each intensity was measured at 1w.

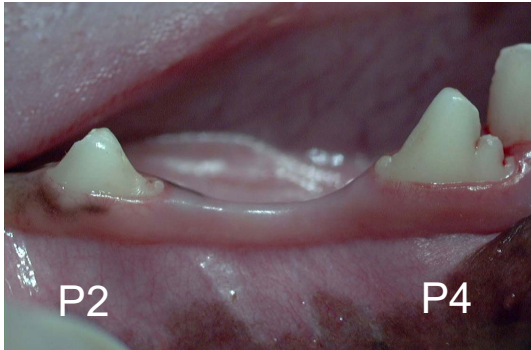
A, C: The values represent cell counts of each protein-expressing cell around the root surface in each section (mean \pm standard deviation; ** $p < 0.01$, * $p < 0.05$; Student t-test; $n = 5$). B, D: The values represent the intensity of each protein expression relative to that in the control (mean \pm standard deviation; ** $p < 0.01$, * $p < 0.05$; Student t-test; $n = 5$).

Fig. S2. BDNF induce osteogenesis/cementogenesis-related genes.

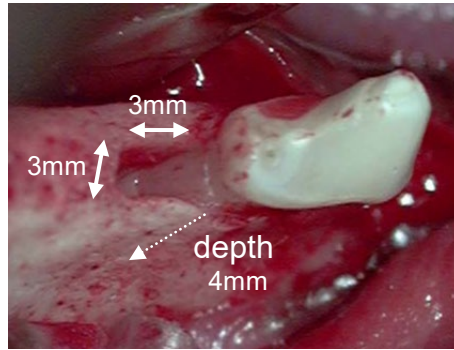
A. hMSCs were treated with BDNF (50 ng/mL) and cultured for 24 hours. B. HPL cells were treated with BDNF (50 ng/mL) and cultured for 24 hours. Runx2, PTPLA1, CEMP1 and SPON mRNA levels determined via real-time PCR. The values represent mRNA expression levels normalized to β -actin mRNA expression relative to that in the control (mean \pm standard deviation; ** $p < 0.01$, * $p < 0.05$; Student t-test; $n = 4$).

Fig. 1

A: Before bone preparation



B: Preparation of 3-wall intrabony defect



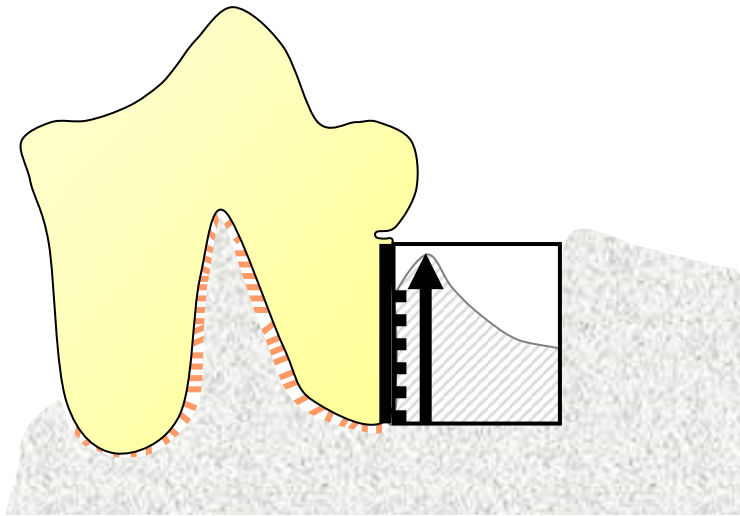
C: Two weeks after bone defect preparation



D: After the application of BDNF



Fig. 2



New cementum formation

$$\frac{\text{■ ■ ■ ■}}{\text{■ ■ ■ ■}} \times 100 (\%)$$

New bone height

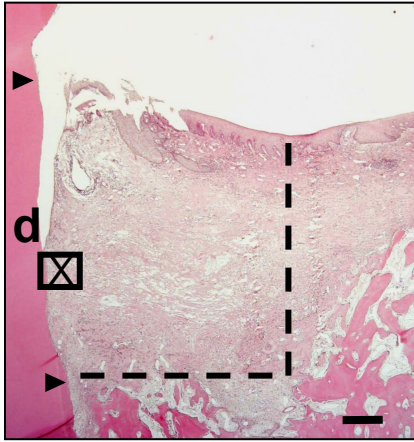
$$\frac{\text{→}}{\text{■ ■ ■ ■}} \times 100 (\%)$$

New bone area

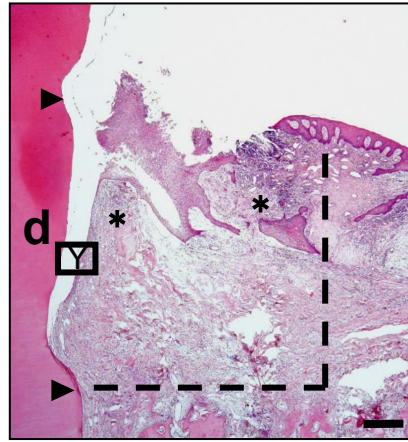
$$\frac{\text{■ ■ ■ ■}}{\text{■ ■ ■ ■}} \times 100 (\%)$$

Fig. 3

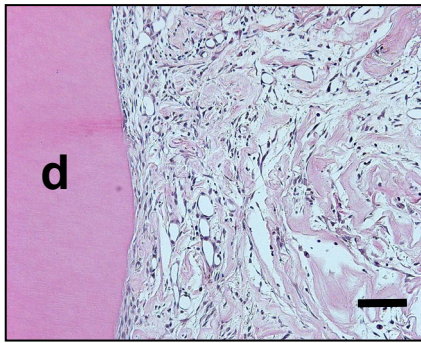
A: BDNF group (HE staining)



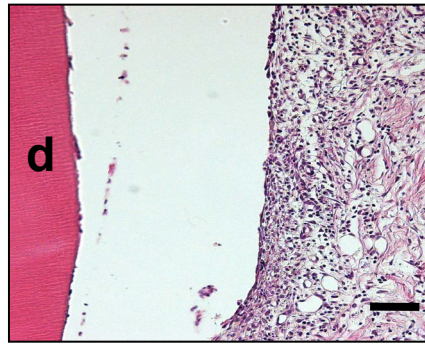
B: Control group (HE staining)



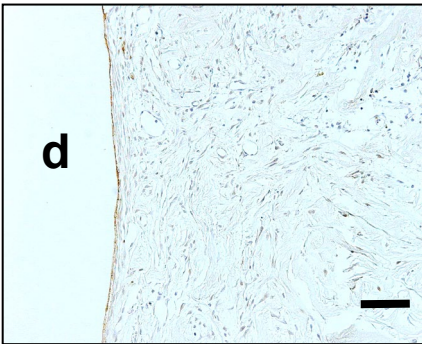
**C: BDNF group
(HE staining, higher magnification)**



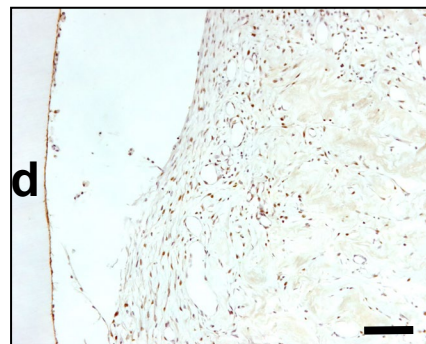
**D: Control group
(HE staining, higher magnification)**



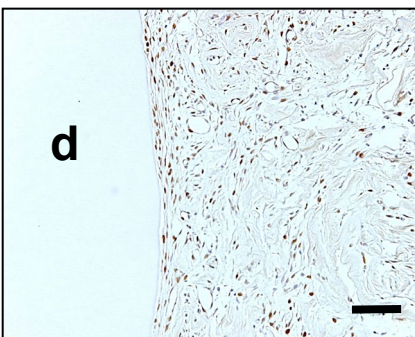
E: BDNF group (OPN staining)



F: Control group (OPN staining)



G: BDNF group (PCNA staining)



H: Control group (PCNA staining)

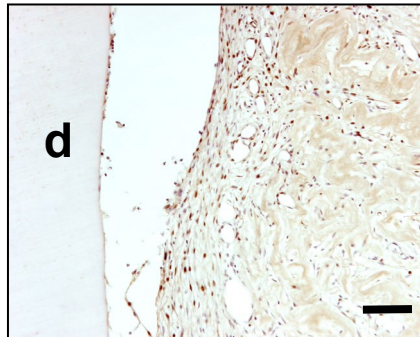
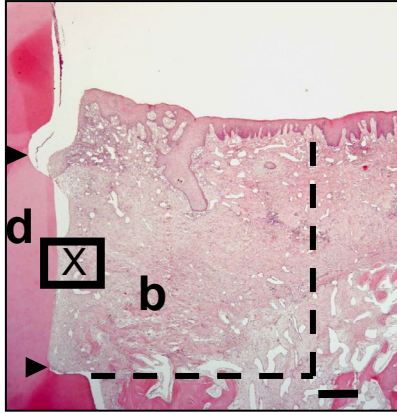
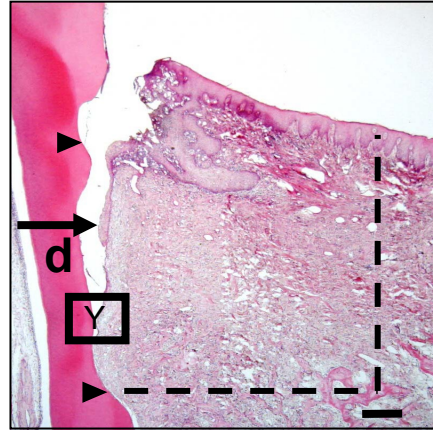


Fig. 4

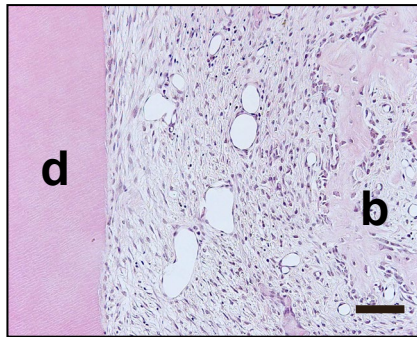
A: BDNF group (HE staining)



B : Control group (HE staining)



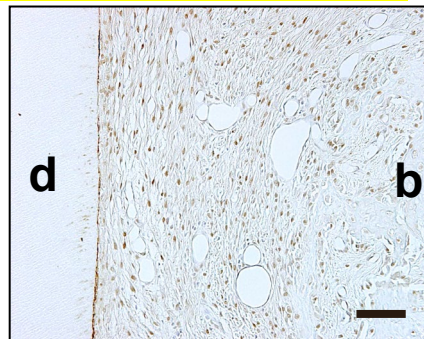
**C : BDNF group
(HE staining, higher magnification)**



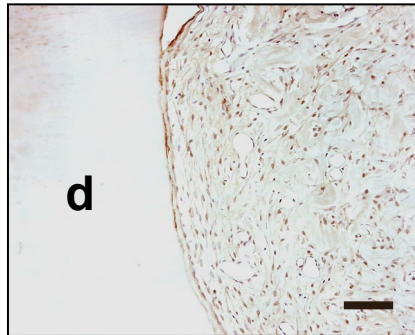
**D : Control group
(HE staining ,higher magnification)**



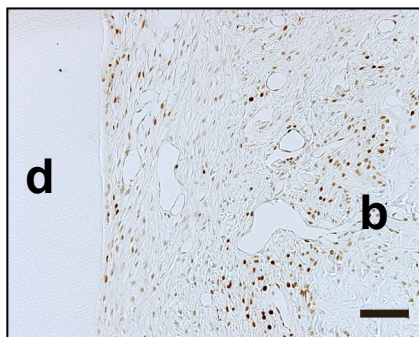
E: BDNF group (OPN staining)



F: Control group (OPN staining)



G: BDNF group (PCNA staining)



H : BDNF group (Azan staining)

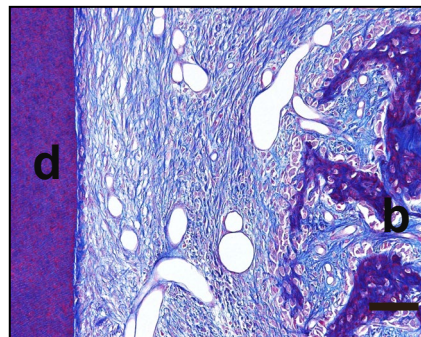
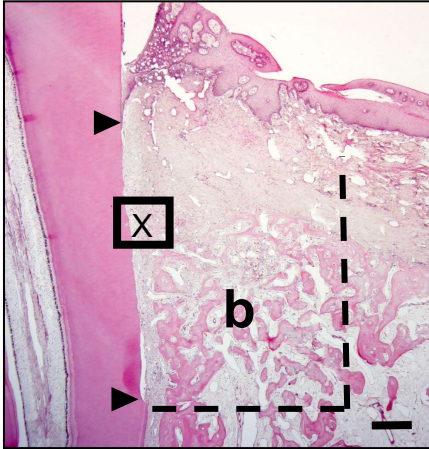
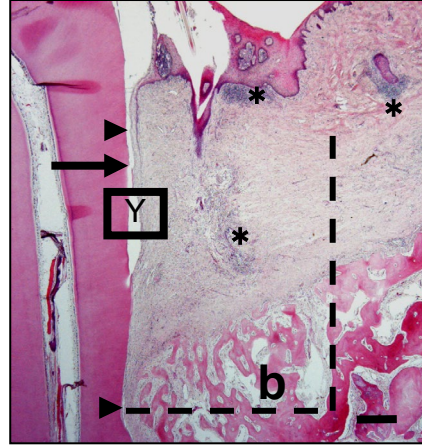


Fig. 5

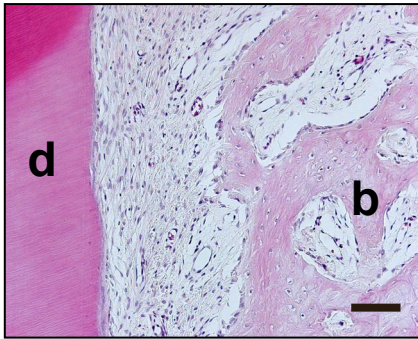
A: BDNF group (HE staining)



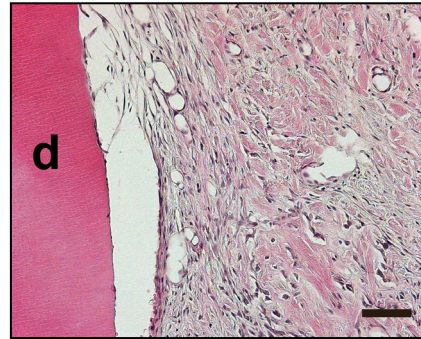
B: Control group (HE staining)



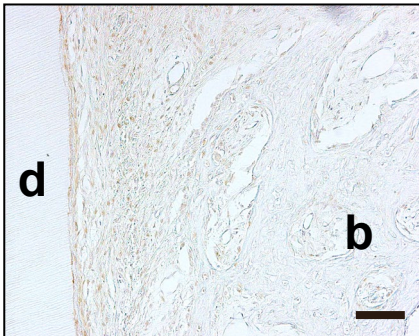
C : BDNF group
(HE staining, higher magnification)



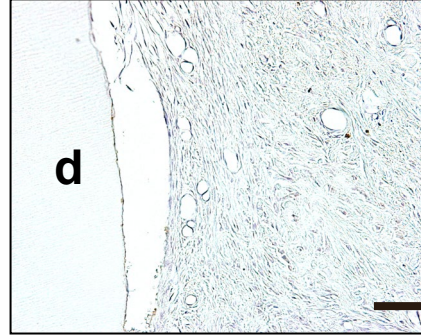
D : Control group
(HE staining ,higher magnification)



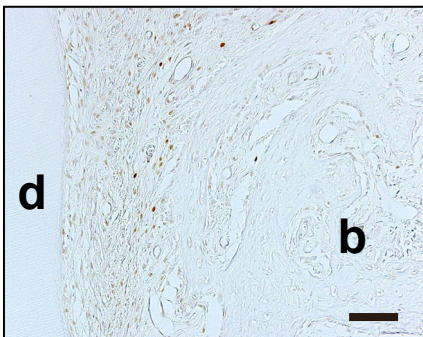
E: BDNF group (OPN staining)



F: Control group (OPN staining)



G: BDNF group (PCNA staining)



H: BDNF group (Azan staining)

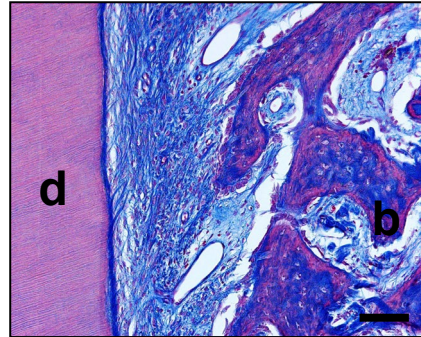


Fig. 6

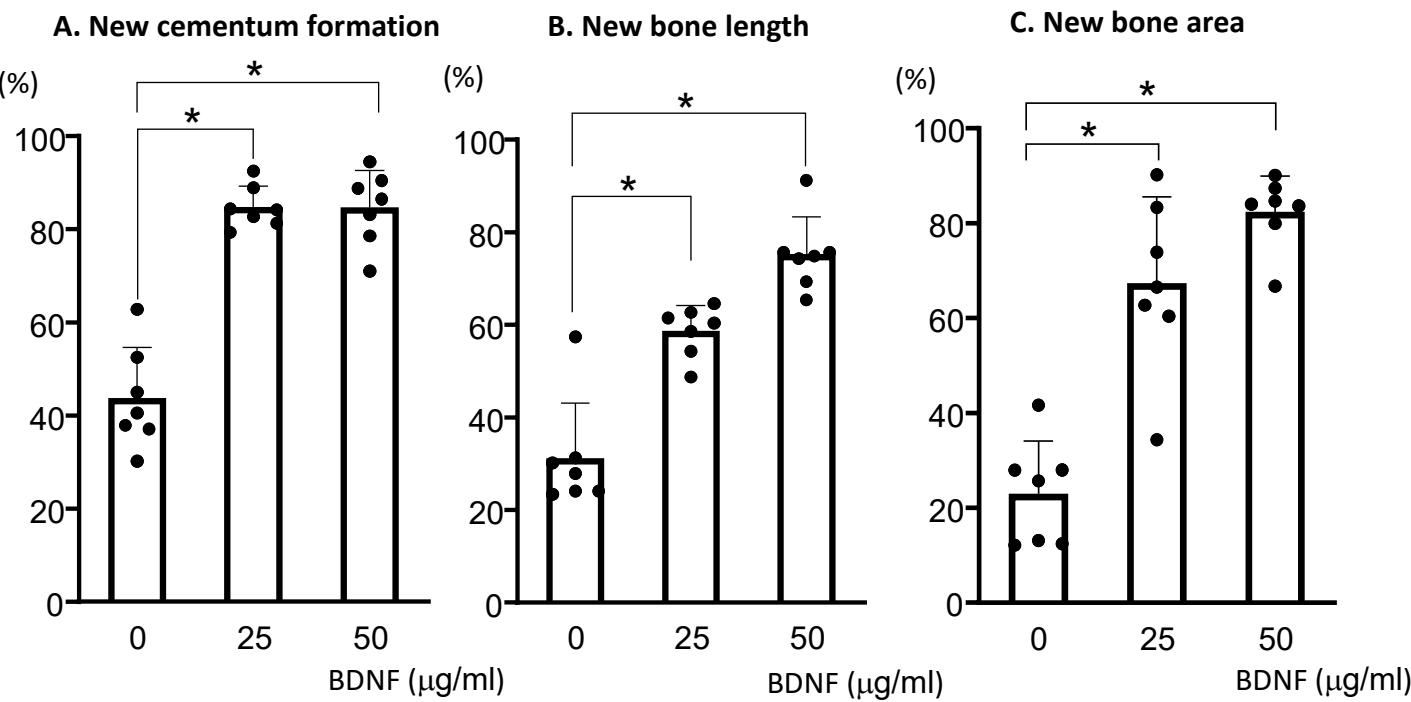
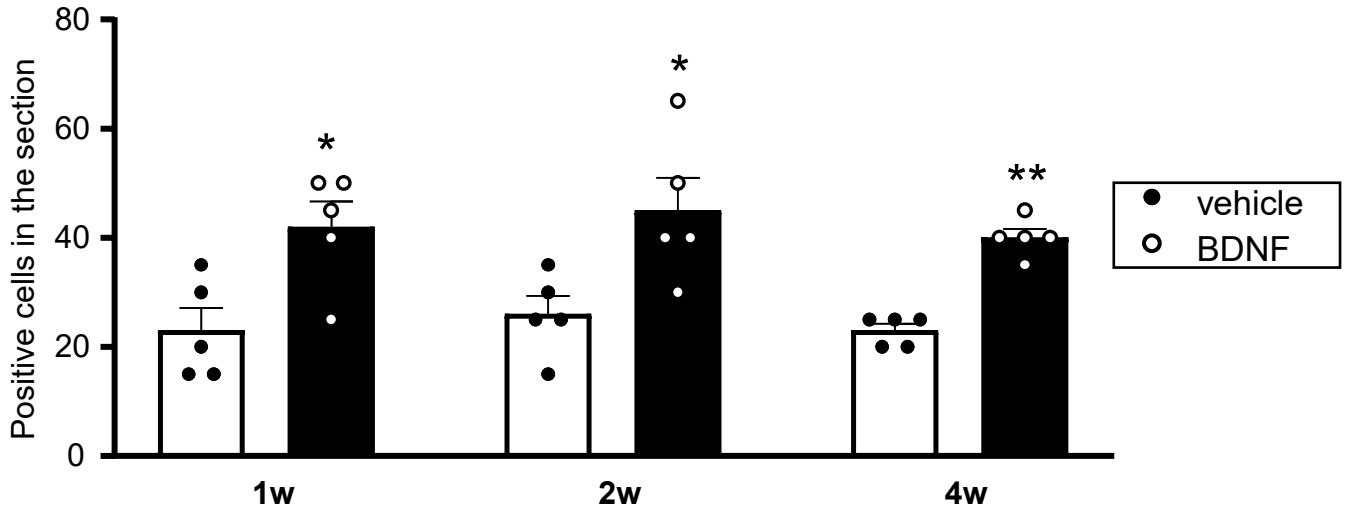
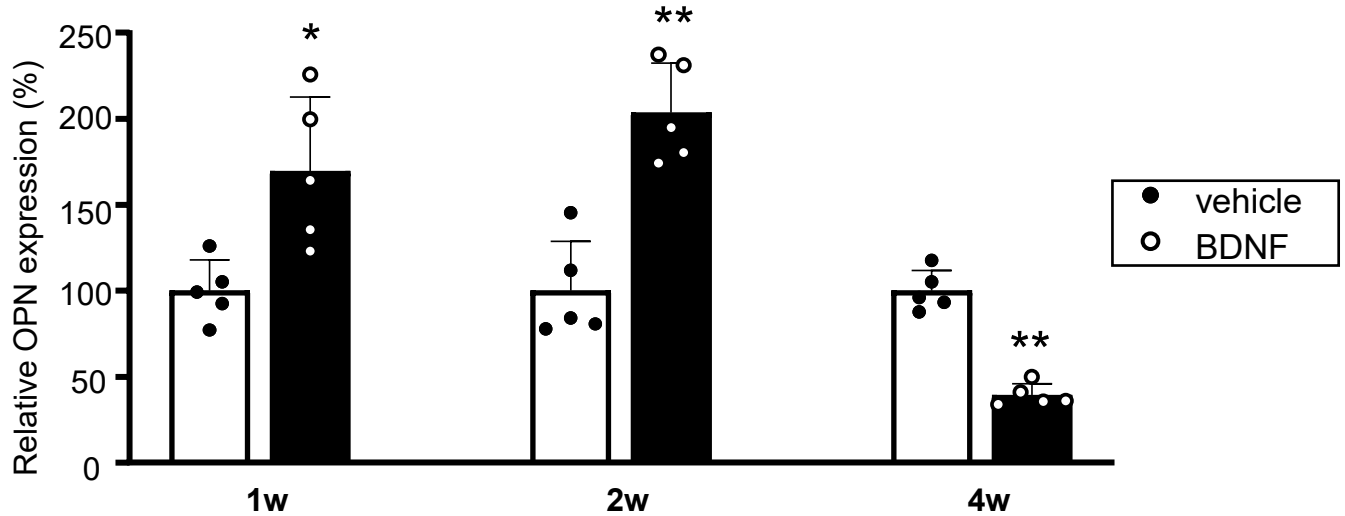


Fig. S1

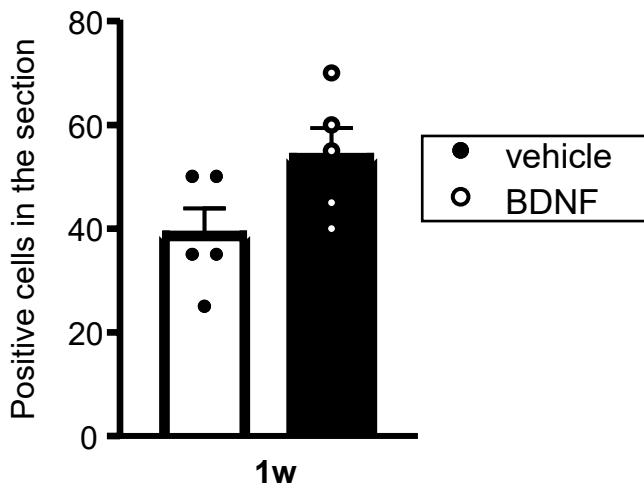
A. OPN expressing cells around the root surface



B. Intensity of OPN expression around the root surface



C. PCNA expressing cells around the root surface



D. Intensity of PCNA expression around the root surface

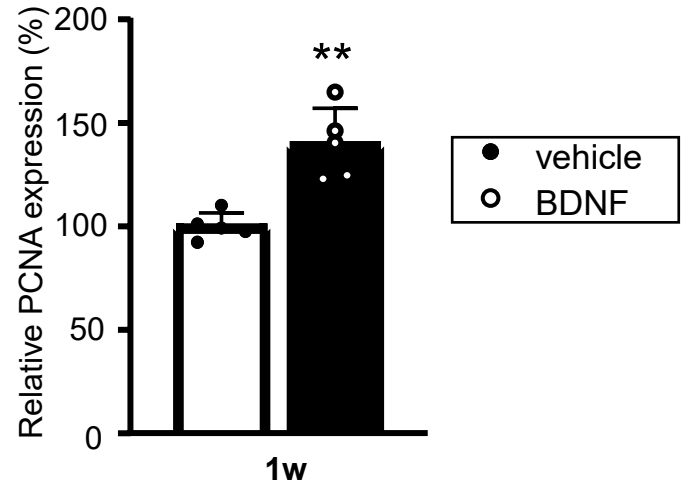
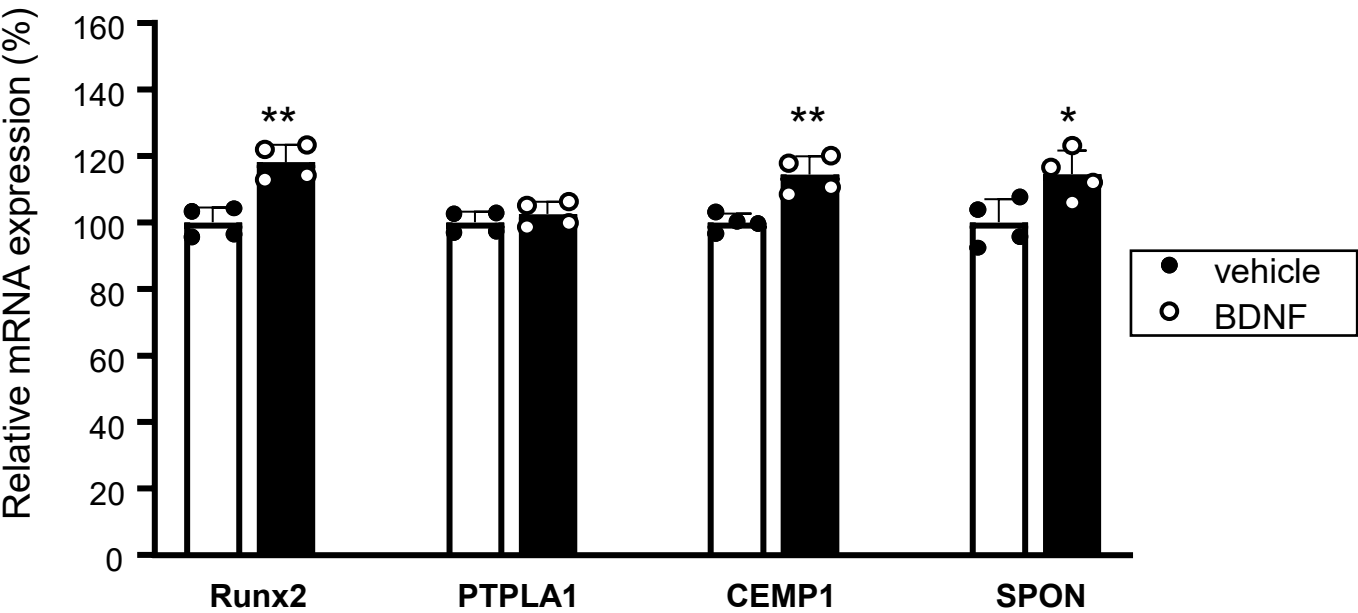


Fig. S2

A.



B.

