Development of Periodontal Tissue Regeneration Therapy with New Bioactive Agents

-Studies on Brain-derived Neurotrophic Factor and Ameloblastin Peptide-

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ABSTRACT

To develop the periodontal regeneration therapy using bioactive agents, effects of two potential agents, brain-derived neurotrophic factor (BDNF) and a synthetic peptide of ameloblastin on growth and differentiation of periodontal ligament cells in vitro and tissue healing in vivo. Both agents enhanced proliferation and mineralization of the periodontal ligament cells and induced periodontal tissue regeneration. We showed that BDNF and the ameloblastin peptide could be used as the promising medication to promote periodontal tissue regeneration.

KEY WORDS: periodontal tissue regeneration, brain-derived neurotrophic factor (BDNF), ameloblastin

INTRODUCTION

The goal of periodontal treatments is regeneration of periodontal tissues that have been lost by periodontal diseases. A periodontal defect after debridement is repopulated by cells from four different sources: oral epithelium, gingival connective tissue, alveolar bone, and periodontal ligament. It is known that new connective tissue attachment can be obtained only when the cells from periodontal ligament cover the denuded root surface. Based on this concept, a new regeneration therapy using a barrier membrane that guides the selective repopulation of periodontal ligament derived cells into a periodontal defect (Takata, 1994) was devised. This treatment procedure is called guided tissue regeneration (GTR) method and widely employed as a predictable regeneration modality. The clinical outcome of the GTR method is right enough for a relatively small defect of periodontal tissues, while predictability for an advanced lesion such as one-wall bony defect and through-and-through furcation involvement is poor. This limited indication of the GTR method depends on limits of the ability of spontaneous proliferation and differentiation of the cells from the remaining periodontal ligament. To overcome the limitation and promote the periodontal regeneration, biological effects of various bioactive agents on migration, growth, differentiation and matrix production of periodontal ligament derived cells have been extensively examined.

In this paper, we describe two studies aiming to develop a new periodontal regeneration therapy using a growth factor, brain-derived neurotrophic factor (BDNF), and a synthetic peptide of an enamel matrix protein, ameloblastin.

1. Periodontal Tissue Regeneration using Brain-derived Neurotrophic Factor

Brain-derived neurotrophic factor (BDNF), cloned as the second member of the neurotrophin family, plays a role in the survival and differentiation of central and peripheral neurons through biding to a product of *trk*B (Barbacid, 1994.; Ebendal, 1992). It has been reported that various types of non-neural cells and tissues express BDNF. Thus, BDNF can regulate functions of non-neural cells as well as neural cells. In the present study, we examined the effect of BDNF on the expression of bone (cementum) related factors and proliferation of human periodontal ligament (HPL) cells. Subsequently, we investigated the effect of BDNF on the regeneration of periodontal tissues in experimental periodontal defects in dogs.

BDNF Increases Bone (Cementum) Related Factors at mRNA and Protein Levels and BrdU Incorporation in HPL Cells

HPL cells were obtained by explant cultures of healthy periodontal ligaments of the mid-root of premolars extracted from patients under orthodontic treatment with their informed consent. BDNF at 50 ng/ml increased mRNA levels of osteopontin (OPN), BMP-2, alkaline phosphatase (ALP) and osteocalcin (OCN) in a dose-dependent manner (Fig. 1A). BDNF also increased secreted OPN and BMP-2 levels in a dose-dependent manner up to 100 ng/ml (Fig. 1B and 1C). Adding BDNF to HPL cells resulted in an increase in procollagen type I C-terminal peptide (PIP) levels in the media (Fig. 1D). BDNF increased BrdU incorporation in HPL cells until 10 ng/ml BDNF. However, at more than 10 ng/ml BDNF the increase in the incorporation declined to the control level (Fig. 1E).

BDNF Enhances Periodontal Tissue Regeneration in Dog

Under general anesthesia with local infiltrated anesthesia, the mandibular second, third and fourth premolars at

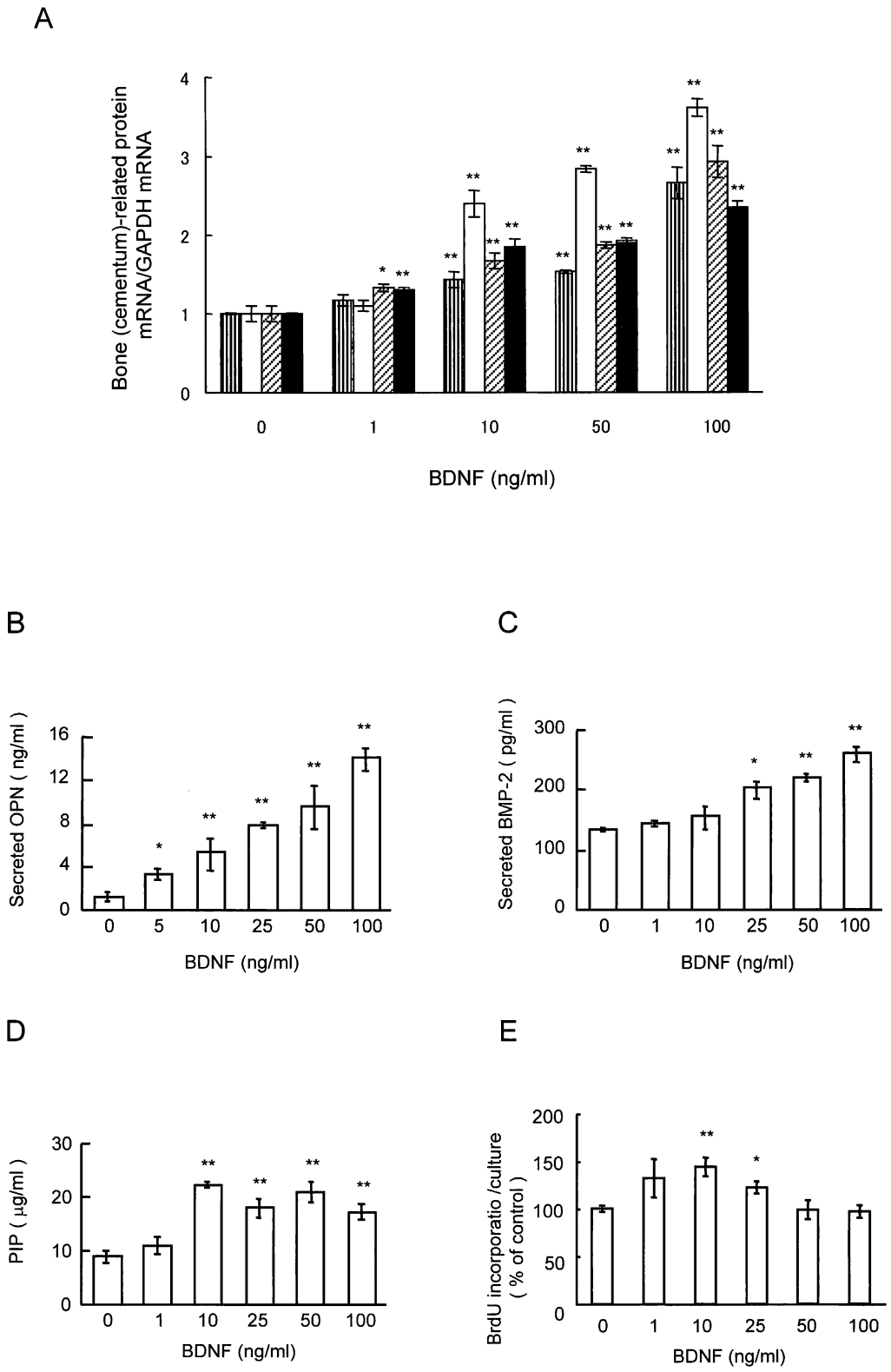

Fig. 1. A. Effects of BDNF on OPN, BMP-2, ALP and OCN mRNA expressions in HPL cells. The B-E. Effects of increasing concentrations of BDNF on the syntheses of OPN (B), BMP-2 (C), PIP (D) and DNA (E) in cultures of HPL cells. Differs significantly (*P<0.05: **P<0.01) from the control.

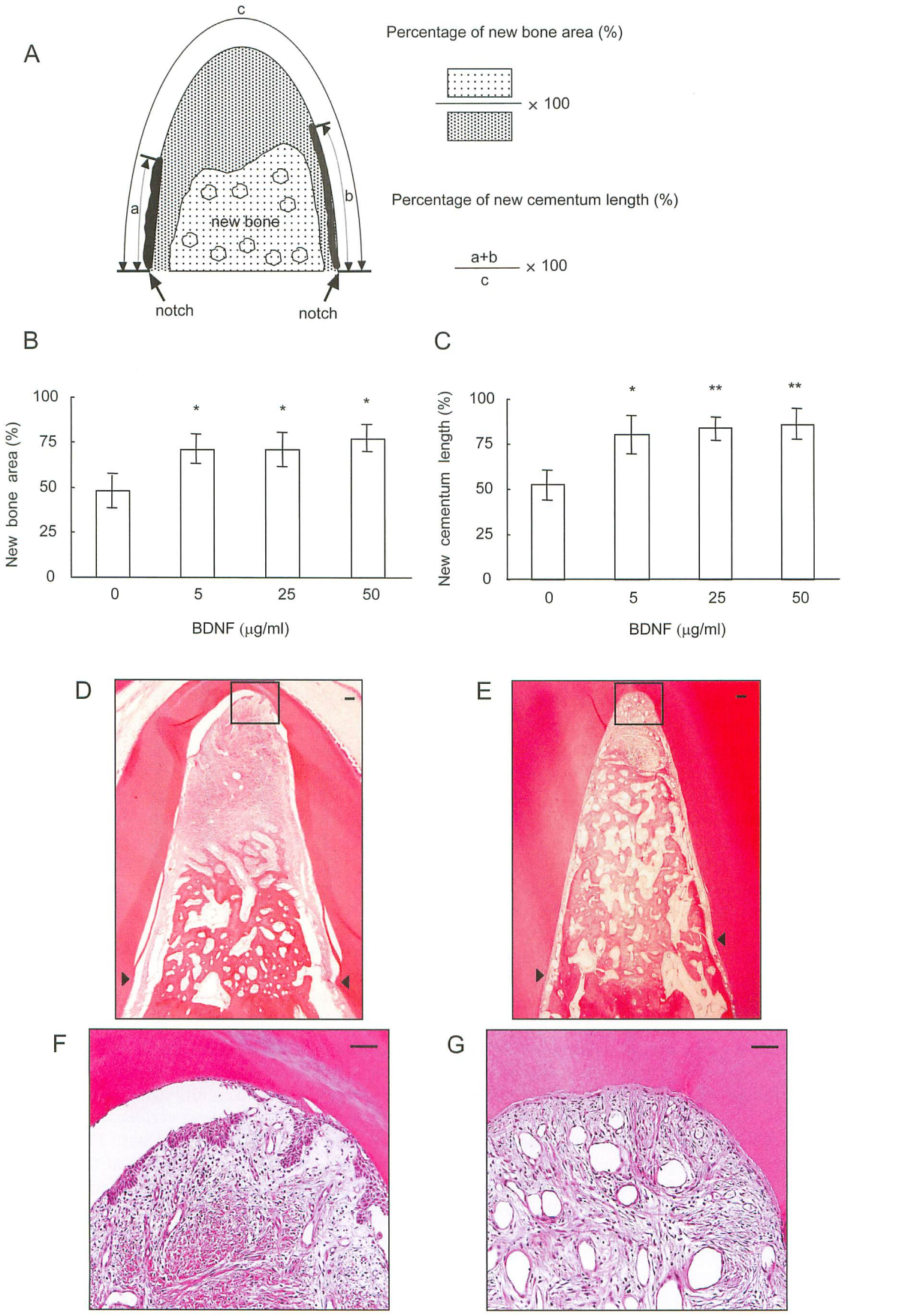

Fig. 2. A. Schematic drawing of the histometric analysis of percentages of new bone area and new cementum length. B and C. Effect of BDNF in experimentally created periodontal defects. The graphs show percentages of new bone area (B) new cementum length (C) by morphometrical analysis. Differs significantly (*P<0.05: **P<0.01) from the control. D and E. Low-power view of furcations representing the control group (D) and BDNF (5 μg/ml) group (E). F and G. Higher magnifications of the rectangular areas shown in D and E, respectively. HE staining.

the right and left sides were utilized for experimentation. Following sulcular incisions, mucoperiosteal flaps were raised and class III furcation defects were surgically created. BDNF (5, 25 and 50 μg/ml) immersed into atelocollagen sponge and applied into the defects. Six weeks after the surgery, histological sections, representing the central portion of the furcation site, were analyzed histologically and morphometrically (Fig 2A). Statistical analysis of the data was performed using ANOVA.

A greater volume of newly formed alveolar bone and a longer newly formed cementum were observed in the BDNF group at 5 μg/ml than in that of the control group (Fig 2B-E). Morphometrical analysis of new bone area showed that BDNF at 5, 25 and 50 μg/ml significantly increased bone area (Fig. 2B) and cementum length (Fig. 2C). Epithelial cells invaded the top of the furcation in the control group and newly formed cementum was not observed in this area (Fig. 2D and F). On the other hand, newly formed cementum was observed on denuded root surfaces of the furcation area and fibers were inserted into the cementum in the BDNF group (Fig. 2E and G). Furthermore, epithelial cell invasion and bone ankylosis were not observed in the BDNF sites (Fig. 2E and G). More blood capillaries were observed in the BDNF group than in the control group and the lamina in the vessels in the BDNF group was larger than in the control group (Fig. 2F and G).

DISCUSSION

BDNF increases expression of ALP, OPN, BMP-2, OCN and type I collagen and DNA synthesis in HPL cells. BDNF is known to be involved in the angiogenesis (Kim et al., 2004). The present in vivo studies showed that BDNF accelerated the formation of periodontal ligament, cementum and alveolar bone and angiogenesis in experimentally created periodontal defects in dogs. Thus, the stimulatory effects of BDNF on the periodontal tissue regeneration were found to result from enhancement of functioning of endothelial cells as well as HPL cells from the in vitro and in vivo studies.

It is noteworthy that ankylosis and epithelial down-growth were not observed in the BDNF group. Ankylosis and epithelial down-growth are not favorable repair phenomena, as they interfere with the completion of periodontal tissue regeneration. Covering the denuded root surface with newly formed cementum may be related to the prevention of ankylosis and epithelial down-growth.

Previous studies showed that fibroblasts engineered ex vivo to secrete BDNF and grafted into a partial cervical hemisection promote axon regeneration while reducing cell loss and atrophy of neurons in the Red nucleus (Liu et al., 2002). Therefore, grafting genetically modified HPL cells that express BDNF as well as BDNF into lost periodontal tissue is a possible new therapy for regenerating and innervating periodontal tissues.

The biological effect of BDNF is mediated through TrkB of high-affinity transmembrane receptor, and the low-affinity receptor of p75, which is a member of the tumor necrosis factor receptor superfamily (Barret, 2000). A truncated receptor lacking the kinase domain has been described for TrkB (Klein et al., 1990). TrkB with and without tyrosine kinase have been detected in neurons, while non-neural cells express both or either form. Our studies showed that HPL cells as well as periodontal ligament tissues expressed TrkB mRNA with and without tyrosine kinase (unpublished data). At present, the mechanism of mediating trophic effects of BDNF via TrkB domain and p75 is still unconfirmed. Therefore, additional studies will be required to clarify the significance of TrkB and p75 in periodontal tissue regeneration.

2. Periodontal Tissue Regeneration using Synthetic Ameloblastin Peptide

Enamel matrix proteins including amelogenin, enamelin, ameloblastin, enamelysin and others play important roles in odontogenesis (Simmmer et al., 2002). Based on the roles of the enamel matrix proteins in odontogenesis, enamel matrix derivatives were applied to periodontal tissue regeneration and good laboratory and clinical results have been obtained (Hammarström, 1997). Thus, it is interesting to decide which protein in EMD is most responsible for the periodontal tissue regeneration.

Ameloblastin is a minor member of enamel matrix proteins that is supposed to act as a messenger molecule for epithelio-mesenchymal interactions in the odontogenesis (Simmer et al., 2002). In the present study, therefore, we examine the effects of ameloblastin in proliferation and differentiation of HPL cells.

Ameloblastin Peptide Stimulates Cell Growth of Periodontal Ligament Cells

HPL cells were plated into a 24 well culture plate (5000 cells/well). After incubation for 24 h (day 0), the culture media were then replaced with a fresh media including the ameloblastin peptide, designed from well-conserved sequences at N-terminal of the protein, at various concentrations (1, 100 and 10000 pg/ml). The number of trypsinized cells was counted using a cell counter at 6 days. Ameloblastin peptide stimulated proliferation of HPL cells in a dose dependent manner (Fig. 3A).

Ameloblastin Peptide Enhances Alkaline Phosphatase and Mineralization Activities of Periodontal Ligament cells

The quantitative analysis of ALP activity was performed biochemically by Bessey-Lowry enzymologic method using nitrophenyl phosphate as a substrate. HPL cells were plated in 24 well culture plates and cultured DMEM with ameloblastin peptide at various concentrations for 1 week. The cells were washed with PBS and homogenized ultrasonically and aliquots of the homogenates were used for quantification of ALP activity. ALP activity was increased by the ameloblastin peptide in a dose dependent manner (Fig. 3B). Mineral nodule formation was detected by Dahl's method for calcium. Cells were placed in a 24 well plate and cultured in DMEM with ameloblastin peptide at various concentrations for 3 weeks. The medium was supplemented with 2 % FBS, 50 μg/ml ascorbic acid and 10 mM sodium β-glycerophosphate. The cells were stained with alizarin red S. The peptide increased mineralized nodule formation in a dose dependent manner (Fig. 3C). The increased ALP

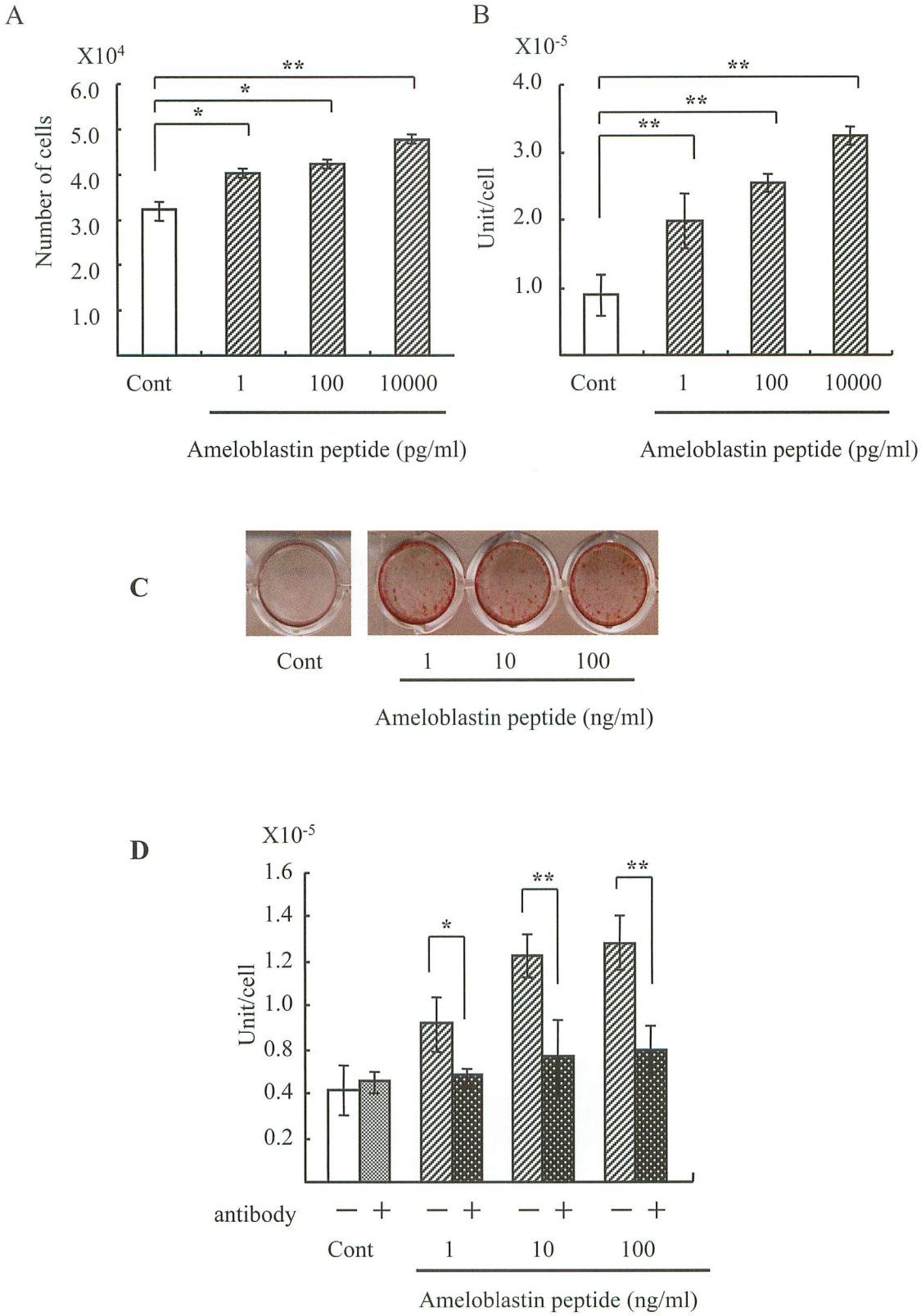

Fig. 3. A. Effect of ameloblastin peptide on proliferation of HPL cells. Cell growth of HPL cells is stimulated in a concentration dependent manner by ameloblastin peptide compared with control culture. *: $P < 0.05$, **: $P < 0.01$, B. Effects of ameloblastin peptide on ALP activity of HPL cells by biochemical method. HPL cells show higher ALP activity than control in a does dependent manner. **: $P < 0.01$, compared with control cultures. C. Alizarin red staining. The peptide increases mineralized nodule formation of in a dose dependent manner D. Effects of anti-ameloblastin antibody on ALP activity of HPL cells. The stimulation of ALP activity in HPL cells by the peptide is significantly inhibited by the anti-ameloblastin antibody. *: $P < 0.05$, **: $P < 0.01$.

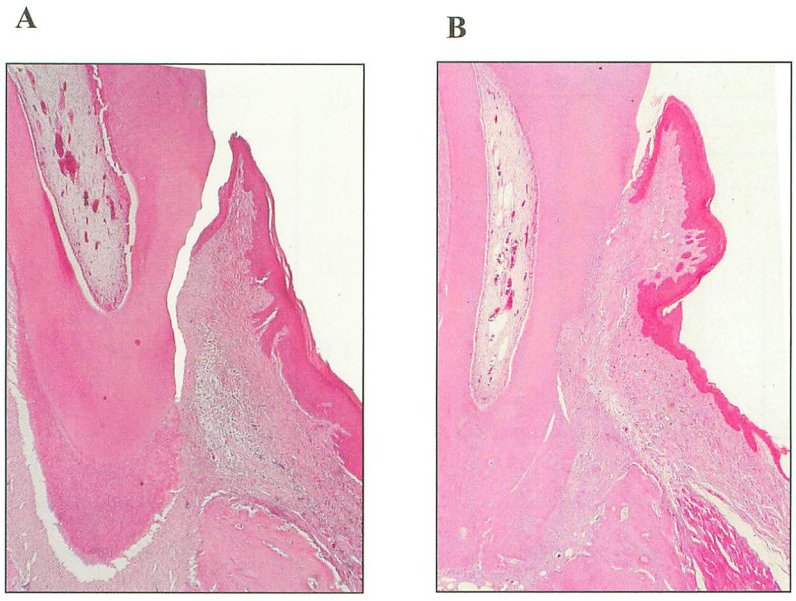

Fig. 4. Effects of the ameloblastin peptide on periodontal tissue regeneration. A. Down-growth of junctional epithelium and separation of gingival tissue from root surface are observed in the control group without the peptide application. B. In the ameloblastin peptide group (0.3mg/ml), down-growth of epithelium is not observed and new connective tissue attachment is formed.

activity induced by ameloblastin was significantly inhibited by an anti-ameloblastin antibody (Fig. 3D).

Ameloblastin Peptide Stimulates Periodontal Tissue Regeneration

Under the abdominal anesthesia, a periodontal tissue defect was experimentally prepared at the mesial root surface of the upper first molar. The defect was applied with 0.3 mg/ml ameloblastin peptide in 1 % hyaluronic acid. At 3 weeks postoperatively, the tissues were prepared for the histological analysis. In the ameloblastin peptide group, the down-growth of epithelium was not observed. And the formation of cementum-like hard tissue with insertion of collagen fiber was formed on the exposed root (Fig. 4A). On the other hand, in control and hyaluronate gel groups, junctional epithelium progressively proliferated apically along the root surface. No connective tissue formation was observed (Fig. 4B).

DISCUSSION

Ameloblastin is expressed on the dentin surface of the root immediately before cementogenesis in a spatiotemporal-specific manner and supposed to act as a messenger molecule for epithelio-mesenchymal interactions in cementogenesis (Simmer et al., 2002; Fukae et al., 2001). In this study, therefore, we examined effects of ameloblastin on cell growth and differentiation of HPL cells and evaluated clinical use of ameloblastin as a novel periodontal regeneration medication. For the study, we did not use the whole length of ameloblastin, but the synthetic peptide of N-terminal region of ameloblastin, because it is very difficult to make recombinant whole amelobastin or to retrieve large amount of ameloblastin from teeth. The region that we synthesized is genetically well conserved among animal species and expected to have important biological properties.

The ameloblastin peptide stimulated the proliferation and differentiation of HPL cells in vitro. Furthermore, the peptide enhanced formation of new connective tissue attachment in vivo experimental model. These results suggest usefulness of the peptide as a treatment agent for the periodontal tissue regeneration.

Recently, we found the possibility that EMD acts via the receptor that possesses the RTK activity on the cell membrane (Matsuda et al., 2002). In addition, since ERK activity is increased following the EMD stimulation, the involvement of a classical signal cascade, RTK-Ras-Raf-MEK-ERK, in some degree was clarified. However, to which receptor the ameloblastin peptide binds and which signal transudation systems the peptide activates must be elucidated in future for further understanding of the biological properties of the peptide and development of signaling-based medication.

SUMMARY

We showed that BDNF and synthetic ameloblastin peptide could be used as a promising medication to promote periodontal tissue regeneration.

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