

## Induction of Bone Morphogenetic Protein-2 from Gingival Epithelial Cells by Oral Bacteria

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We hypothesized that plaque-associated bacteria may have a role in maintenance of alveolar bone. To test it, immortalized gingival epithelial HOK-16B cells were co-cultured with live or lysed eight plaque bacterial species and the expression levels of bone morphogenetic protein (BMP)-2 and -4 were examined by real time reverse transcription-polymerase chain reaction. Un-stimulated HOK-16B cells expressed both BMP-2 and -4. Co-culture with plaque bacterial lysates had significant effects on the level of BMP-2 but not on that of BMP-4. Five species including *Streptococcus sanguinis*, *S. gordonii*, *Veillonella atypica*, *Porphyromonas gingivalis*, and *Treponema denticola* substantially up-regulated the level of BMP-2. In contrary to the upregulatory effect of lysate, live *T. denticola* suppressed the expression of BMP-2. In addition, *in vitro* osteoblastic differentiation assay using C2C12 cells and the conditioned medium of HOK-16B cells confirmed the production of BMPs by gingival epithelial cells and the modulation of BMP expression by the lysates of *S. sanguinis* and *T. denticola*. In conclusion, we have shown that plaque bacteria can regulate the expression of BMP-2 by gingival epithelial cells, the physiologic meaning of which needs further investigation.

**Key words:** oral bacteria, gingival epithelium, BMP, osteoblast

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### Introduction

Oral cavity is endowed with characteristics to be an excellent habitat for various bacteria. First, there are diverse surfaces for microbial colonization, such as hard tissue, non-keratinized, keratinized, and specialized oral mucosa. Second, these surfaces are constantly bathed in fluid, mostly saliva. Third, rich nutrients are provided from diet, desquamated epithelial cells, saliva, and gingival crevicular fluid. Therefore, as many as 700 bacterial species can colonize in oral cavity (Paster et al., 2006). Among these, only a score of species are associated with diseases such as dental caries, periodontitis, and infectious endocarditis. Most other oral bacteria have been considered to coexist with host peacefully without harm or obvious benefit, playing only a passive role through competition with pathogenic microbes for colonization sites and nutrients. Recently, new beneficial roles of gut microflora have been reported: it fortifies the barrier function of epithelia, enhances energy harvest, and induces the maturation of peripheral lymphoid organs (Hooper et al, 2001; Mazmanian et al., 2005; Turnbaugh et al., 2006). It suggests that oral microbiota may also have a new role other than mere competition.

Adult bone tissues are continuously remodeled, and bone morphogenetic proteins (BMPs), especially BMP-2, -4, and -7 have important roles in the homeostasis of the bone tissues (Canalis et al., 2003). Alveolar ridge, the supporting bone of tooth, gradually decreases after tooth loss. Although disuse atrophy due to the loss of mechanical stimulation is a current theory to explain this phenomenon (Pavlin and Gluhak-Heinrich, 2001), it does not explain well why denture-transmitted mechanical stimulation does not protect from the alveolar bone loss, while implant therapy maintains the volume of alveolar bone. Penetration of tooth through oral

mucosa creates gingival sulcus, a unique anatomic structure at the junction of tooth and epithelia. This area provides bacteria with both hard and soft surfaces for colonization, protection from natural mechanical clearance, anaerobic environment, and the nutritionally-rich gingival crevicular fluid (Socransky and Haffajee, 2005). In consequence, dental plaque harboring diverse and abundant bacteria is formed in gingival sulcus. A few studies in literature indicate that tooth loss causes change in microflora surrounding alveolar ridge (Kononen et al., 1991; Danser et al., 1997; Socransky and Haffajee, 2005). In contrast, peri-implant microflora is similar to that of dental plaque, probably due to the re-creation of gingival sulcus (Lee et al., 1999; Quirynen et al., 2006; Leonhardt et al., 2002). Therefore, we hypothesized that dental plaque-associated bacteria may have a role in maintenance of alveolar bone. To test this hypothesis, we studied the effect of various oral bacteria on the expression of BMP-2 and -4 by gingival epithelial cells and have shown that some oral bacteria can induce osteogenic activity from gingival epithelial cells.

## Materials and Methods

### Bacterial strains and culture

*Streptococcus sanguinis* NCTC 10904, *S. gordonii* ATCC 10558, *Veillonella atypica* ATCC 17744, *Fusobacterium nucleatum* ATCC 10953, *Prevotella intermedia* ATCC 25611, *Porphyromonas gingivalis* ATCC 49417, *Tannerella forsythia* ATCC 43407, and *Treponema denticola* ATCC 33521 strains obtained from ATCC (Bethesda, U.S.A.) were cultured as described previously (Ji et al., 2007a). Bacteria in the log phase were harvested and washed twice with DPBS. Part of the bacterial suspension was stained with 5  $\mu$ M SYTOX-Green nucleic acid stain (Molecular probes, Eugene, U.S.A.), and the concentration and viability of bacteria were determined by flow cytometry (Ji et al., 2005). A fraction of the bacterial suspension with determined concentration was sonicated (Sonic Dismembrator 300, Fisher Scientific, Fair Lawn, U.S.A.) for 15 min on ice-water to prepare lysates. The breakage of bacterial cell was confirmed by examination under a light microscope. The concentration of protein in the bacterial lysates was determined using a BCA kit (Pierce, Rockford, U.S.A.).

### Epithelial cell culture

HOK-16B was obtained from Dr. N-H. Park (University of California Los Angeles, Los Angeles, U.S.A.), and maintained in keratinocyte growth medium (KGM, Clonetics, San Diego, CA) containing 0.15 mM calcium and a supplementary KGM bullet kit that includes antibiotics GA-1000 (gentamicin and amphotericin B) and growth factors.

### Infection of epithelial cells with oral bacteria

HOK-16B cells were plated at  $6 \times 10^4$  cells/500  $\mu$ L/well in

triplicate into 24-well plates one day before infection. At 80% confluence, cells were infected with eight different species of live or lysed bacteria in KGM containing 2% heat-inactivated human sera (Sigma, Saint Louis, U.S.A.) and cultured at 37°C in a water-saturated atmosphere of 95% air and 5% CO<sub>2</sub> for 24 h to simulate chronic stimulation *in vivo*. Aerobic condition was chosen for the co-culture of bacteria with HOK-16B cells, since culture under anaerobic condition was too stressful to HOK-16B cells. To prevent the outgrowth of facultative anaerobic bacteria, the antibiotics in KGM were maintained. Under these conditions, 35-60% of bacteria survived without bacterial growth (Ji et al., 2007b). The multiplicities of infection (MOI) of 1000 were based on the total number of bacteria. The concentrations of the bacterial lysates are based on the protein concentration. Total RNA was extracted from combined cells of triplicate wells using TRIzol (Invitrogen, Carlsbad, U.S.A.). Supernatant of culture medium was stored at -80°C until use for osteoblastic differentiation assay. Experiments were repeated two times. In the separate preliminary experiments, the concentration and viability of bacteria were re-analyzed after co-culture. In addition, the effect of bacteria on HOK-16B cell viability was determined by flow cytometry after staining with trypan blue, as previously described (Busetto et al., 2004). All bacterial species had no cytotoxic effect on HOK-16B cells up to MOI 2000.

### Real time reverse transcription polymerase chain reaction (real time RT-PCR)

Total RNA (2  $\mu$ g) was subjected to reverse transcription with (dT)<sub>18</sub> and Superscript II enzyme (Invitrogen) in a 25  $\mu$ L reaction mix at 42°C for 1 h. The expression levels of BMP genes were measured semi-quantitatively by real-time RT-PCR. Real time-PCR was performed in a 20  $\mu$ L reaction mix containing 1  $\mu$ L template cDNA, SYBR Premix Ex *Taq*, ROX Reference Dye (Takara Bio, Otsu, Japan), and each primer (0.2  $\mu$ M). The primer sequences used are as followings: 5'-GGAAGA ACTACCAGAAACGAG-3' and 5'-AGATGATCAGCCAGAGGAAAA-3' for BMP-2; 5'-ACCTGAGACGGGAAGAAAA-3' and 5'-TTAAAGAGGAAACGAAAAGCA-3' for BMP-4; and 5'-CAGCCTCAAGATCATCAGCA-3' and 5'-CCATCCACAGTCTTCTGGGT-3' for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Amplification was performed in a fluorescence thermocycler (Applied Biosystems 7500 Real-time PCR, Foster City, U.S.A.) under the following conditions: initial denaturation at 94°C for 1 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 15 s, and elongation at 72°C for 33 s. The specificity of the PCR product was verified by melting curve analysis and examination on a 2% agarose gel. The housekeeping gene, GAPDH, was amplified in parallel with the gene of interest. Relative copy numbers compared to GAPDH were calculated using 2<sup>- $\Delta$ Ct</sup>. Real-Time PCR was performed in triplicate for each RNA sample. The mean  $\pm$  SEM values of the relative copy numbers from six

assays were expressed as induction fold, compared to that of control culture without bacteria.

### Osteoblastic differentiation assay

C2C12 myoblast cell line (ATCC) that can be differentiated into osteoblasts on BMPs was seeded at a density of  $5 \times 10^4$  cells per well in a 48-well plate, and cultured in  $\alpha$ -MEM containing 5% FBS and 12.5% conditioned medium of HOK-16B cells for 7 days. After culture, the cells were fixed with 4% paraformaldehyde and subjected to alkaline phosphatase staining using a kit (Sigma).

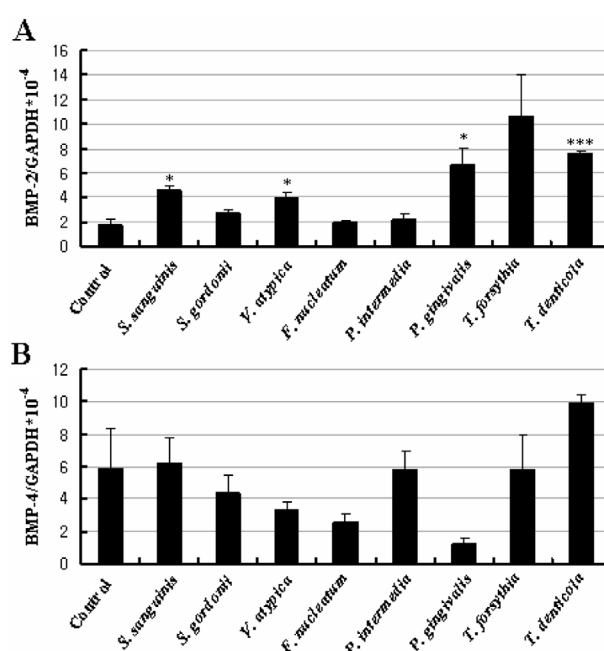
### Statistics

Statistics was performed using the relative copy numbers of BMPs to determine the difference between control and cells co-cultured with each bacterial species. In the mean time, induction rates were used to determine the difference in the responses induced by live versus lysed bacteria. The differences between the two groups were analyzed with the two-tailed non-paired Student's *t*-test. Data were considered statistically significant at  $p < 0.05$ .

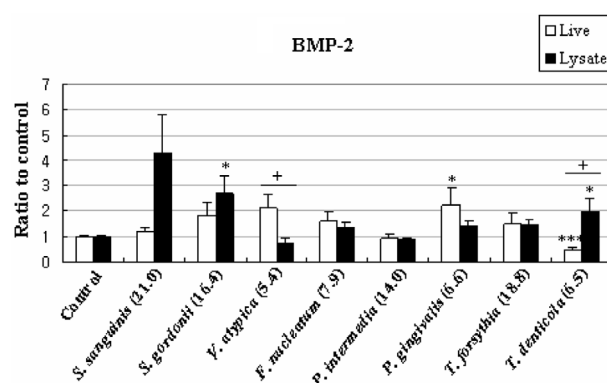
## Results

HOK-16B is an immortalized cell line established by the transfection of human papillomavirus type 16 DNA into primary human oral keratinocytes cultured from excised gingival tissue (Park et al., 1991). To explore the potential role of plaque bacteria in maintenance of alveolar bone through epithelia, HOK-16B cells were co-cultured with the lysates of eight plaque bacterial species, and then the expression levels of BMP-2 and -4 were evaluated by real time RT-PCR. Un-stimulated HOK-16B cells expressed both BMP-2 and -4. Co-culture with plaque bacteria had various effects on the levels of BMP-2 and -4, from up- to down-regulation. Four bacterial species including *S. sanguinis*, *V. atypica*, *P. gingivalis*, and *T. denticola* significantly up-regulated the level of BMP-2 expression (Fig. 1A). Although the level of BMP-4 expression was increased by *T. denticola* and decreased by *S. gordonii*, *V. atypica*, *F. nucleatum*, and *P. gingivalis*, no statistical significance was observed (Fig. 1B).

Live bacteria and bacterial debris present in dental plaque may induce different responses from epithelial cells, since some oral bacteria can invade epithelial cells and may inject bacterial substances directly into the cytoplasm of a host cell (Abrahams et al, 2006; Lamont et al., 2002; Madden et al., 2001). In order to examine the differential effect of live bacteria vs. bacterial product on the induction of BMPs by epithelial cell, HOK-16B cells were co-cultured with live or lysed bacteria at the MOI of 1000, and the level of BMP-2 expression was examined. The pattern of BMP-2 induction by bacterial lysate at the MOI 1000 was quite comparable to that obtained by the lysate 50  $\mu$ g/ml in figure 1, considering

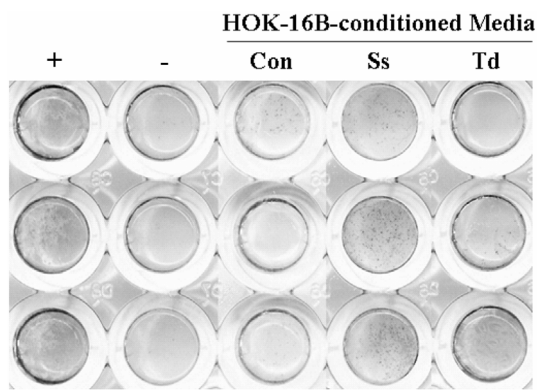


**Fig. 1.** Effect of various bacterial lysates on the expression of BMP-2 and -4 by gingival epithelial cells. HOK-16B cells were co-cultured with bacterial lysates (50  $\mu$ g/ml based on protein concentration) for 24 h. Expression of BMP-2 (A) and -4 (B) by HOK-16B cells was evaluated by real time RT-PCR. Each column represents the mean  $\pm$  SEM of the relative copy numbers of gene of interest compared to those of GAPDH gene. \*,  $p < 0.05$ , \*\*\*,  $p < 0.001$ , versus control.



**Fig. 2.** Effect of live or lysed bacteria on the expression of BMP-2 by gingival epithelial cells. HOK-16B cells were co-cultured with live or lysed bacteria at MOI 1000 for 24 h. The numbers in parentheses represent the protein concentration ( $\mu$ g/ml) of the lysates at MOI 1000. Expression of BMP-2 by HOK-16B cells was evaluated by real time RT-PCR. The mean  $\pm$  SEM values of the relative copy numbers were expressed as induction fold, compared to those of control culture without bacteria. \*,  $p < 0.05$ , \*\*\*,  $p < 0.001$  versus control. +,  $p < 0.05$ , versus live bacteria

the amount of the lysate used. Live bacteria sometimes induced responses different from those induced by bacterial lysate (Fig. 2, marked by +). Live *V. atypica* induced BMP-2 more efficiently than lysed one. Live *T. denticola* significantly suppressed the expression of BMP-2, contrary to the effect



**Fig. 3.** Effect of HOK-16B-conditioned medium on osteoblastic differentiation. C2C12 cells seeded at  $5 \times 10^4$  cells/well in a 48-well plate were cultured in  $\alpha$ -MEM containing 5% FBS and 12.5% conditioned medium of HOK-16B cells for 7 days. After the culture, the cells were fixed with 4% paraformaldehyde and subjected to alkaline phosphatase staining. +, a positive control cultured in the presence of 300 ng/ml BMP-2. -, a negative control cultured with  $\alpha$ -MEM alone. Con, control culture of HOK-16B. Ss, HOK-16B co-cultured with the lysate of *S. sanguinis* at MOI 1000. Td, HOK-16B co-cultured with the lysate of *T. denticola* at MOI 1000.

by its lysate.

The production of BMPs by HOK-16B cells and the modulation of BMP expression by plaque bacteria were confirmed by *in vitro* osteoblastic differentiation assay. The pluripotent mesenchymal cell line C2C12 cells can be induced to differentiate into osteoblasts by BMP-2 (Nishimura et al., 1998). Conditioned medium of HOK-16B cells alone induced slight expression of alkaline phosphatase, the marker gene of osteoblasts, from C2C12 cells. Conditioned medium of HOK-16B cells co-cultured with the lysates of either *S. sanguinis* or *T. denticola* substantially increased the expression of alkaline phosphatase, confirming the results of real time RT-PCR.

## Discussion

In this study, we have shown that gingival epithelial cells express BMPs and plaque bacteria modulate their expression.

Among the eight bacterial species studied, five species except *F. nucleatum*, *P. intermedia*, and *T. forsythia* up-regulated the level of BMP-2. It is quite different from the induction pattern of antimicrobial peptides and IL-8 that were induced by *F. nucleatum* and *P. intermedia* most efficiently (Ji et al., 2007b). When the effect of bacterial lysates on BMP-2 expression at the same MOI was compared, early colonizers such as *S. sanguinis* and *S. gordonii* were more efficient inducer of BMP-2 than late colonizers (Fig. 2). It coincides with the observation that these two species are compatible with periodontal health (Feng and Weinberg, 2006). Live *T. denticola* suppressed the expression of BMP-2, while its lysate significantly enhanced BMP-2. Live but not lysed *T. denticola* significantly

suppressed HBD-1 transcription and IL-8 accumulation as well (Ji et al., 2007b). Therefore, live *T. denticola* seems to have ability to down-regulate variety of host genes. It is interesting that the lysate of *T. denticola* known as periodontopathogen enhances the expression of BMP-2 by gingival epithelial cells. Induction of osteoclastogenesis by the lysate of *T. denticola* in the co-culture system of primary osteoblasts and bone marrow cells has been reported (Choi et al., 2003). The effect of *T. denticola* on alveolar bone homeostasis may be determined by the efficiency of bacterial killing in gingival sulcus by host defense system, penetration of bacteria and/or bacterial products into alveolar bone surface, BMPs produced by epithelial cells, or net effects of all these.

BMP-2 and -4 have potent osteogenic activity (Lian et al., 2006). In this paper, we have shown that gingival epithelial cells produce BMPs and oral bacteria can regulate the expression of BMPs by the gingival epithelial cells. However, further studies are required to have a complete answer to our original hypothesis. It is clear that tooth loss causes change in microflora, but exact difference between the microflora of dental plaque and that on the oral mucosa of edentulous alveolar ridge is not known. Comparison of microbial composition of biofilm on teeth and various oral soft tissues from healthy people revealed that the increased proportions of the *Actinomyces* species colonized tooth surface, while the proportions of *S. oralis* and *S. mitis* were increased in samples from soft tissues (Mager et al., 2003). Study on the effect of *Actinomyces* species, *S. oralis* and *S. mitis* on the expression of BMPs by epithelial cells may further clarify the potential role of oral bacteria in alveolar bone homeostasis through the regulation of BMP expression. Comparison of alveolar bone density of normal vs. germ-free mice would be also useful.

Although BMPs are well-known as osteogenic growth factors, their roles in a number of non-osteogenic developments have been shown. BMPs act as signals of epidermal induction, and inhibition of the BMP signaling pathways leads to neural induction during embryogenesis (Casellas et al., 1998). In addition, BMPs are involved in epithelial and smooth muscle differentiation, mammary gland development, and stomach gland formation (Chadalavada et al., 2005; Narita et al., 2000; Phippard et al., 1996). Therefore, BMPs expressed by gingival epithelial cells may have a role in the regulation of epithelial differentiation and wound healing.

In conclusion, we have shown that plaque bacteria can regulate the expression of BMP-2 by gingival epithelial cells, the physiologic meaning of which needs further investigation.

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