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Original

High concentration of sodium butyrate suppresses osteoblastic differentiation and mineralized nodule formation in ROS17/2.8 cells

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Abstract: Periodontitis is a destructive disease that is likely the result of the activities of different microbial complexes, including anaerobic Gram-negative periodontopathic bacteria. Butyric acid (sodium butyrate; BA) is a major metabolic by-product of anaerobic Gram-negative periodontopathic bacteria present in subgingival plaque. This study was undertaken to examine the effect of BA on the expression of osteogenesis-related transcription factors and mineralized nodule formation in osteoblastic ROS17/2.8 cells. The cells were cultured with 0 (control), 10^{-5}, 10^{-4}, or 10^{-3} M BA for up to 7 days. The gene and protein expression levels of transcription factors such as Runx2, Osterix, Dlx5, Msx2, and AJ18, as well as extracellular matrix proteins such as bone sialoprotein (BSP) and osteocalcin, were examined using real-time PCR and Western blotting, respectively. Mineralized nodule formation was detected by alizarin red staining. The expression of Runx2, Osterix, Dlx5, and Msx2 decreased significantly in the presence of 10^{-3} M BA compared to the control, whereas AJ18 expression increased significantly. Mineralized nodule formation decreased markedly in the presence of 10^{-3} M BA. Alkaline phosphatase activity and the expression of bone sialoprotein and osteocalcin decreased significantly in the presence of 10^{-3} M BA compared to the control. These results suggest that 10^{-3} M BA suppresses osteoblastic differentiation and mineralized nodule formation in ROS17/2.8 cells. (J Oral Sci 53, 509-516, 2011)

Keywords: butyric acid; mineralized nodule formation; osteoblasts; osteogenesis-related transcription; periodontitis.

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Introduction

Periodontitis, one of the most common chronic inflammatory diseases in humans, is caused by anaerobic Gram-negative periodontopathic bacteria, such as *Porphyromonas*, *Prevotella*, and *Fusobacterium*, in subgingival plaque. Short-chain carboxylic fatty acids (SCFAs) such as acetic acid, propionic acid, and butyric acid (sodium butyrate, BA) are a major by-product of the metabolism of anaerobic Gram-negative periodontopathic bacteria and are detected in gingival crevicular fluid from human periodontal pockets (1). The amount of SCFAs in the crevicular fluid has been correlated with the extent of periodontal breakdown in chronic adult periodontitis, and a possible role for SCFAs in the pathogenesis of periodontal disease has been hypothesized (1,2). Periodontopathic bacteria produce a high amount of BA, which is reported to inhibit gingival fibroblast proliferation (3-5), the stimulation of T-cell apoptosis (6-11), and osteoblast maturation (12,13).

Osteoblasts perform a central role in bone formation via high alkaline phosphatase (ALPase) activity and the production of extracellular matrix proteins such as type I collagen, bone sialoprotein (BSP), and osteocalcin. Osteoblastic differentiation is controlled by multiple

Table 1 PCR primers used in the experiments

Target	Forward Primer	Reverse Primer	GenBank Acc No.
Runx2	5'-CGCATTCCTCATCCCAGTAT-3'	5'-GCCTGGGGTCTGTAATCTGA-3'	NM_053470
AJ18	5'-GTGATTGGCAAGCTGCAGAA-3'	5'-TAGCAGCCCACGAGATGGTC-3'	AJ833597
Osterix	5'-GCCAGAAGCTGTGAAACCTC-3'	5'-GCTGCAAGCTCTCCATAACC-3'	NM_181374
Dlx5	5'-GCGCTCAACCCATACCAGT-3'	5'-ACTCGGGACTCGGTTGTAGG-3'	NM_005221
Msx2	5'-TCACCACGTCCCAGCTTCTAG-3'	5'-AGCTTTTCCAGTTGCGCCTCC-3'	NM_012982
Osteocalcin	5'-GGTGCAGACCTAGCAGACACCA-3'	5'-AGGTAGCGCCGGAGTCTCTATTCA-3'	M25490
BSP	5'-TGTGGAATGGTGCTACGGT-3'	5'-CATCAACAGCCCTGATTTA-3'	NM_012587.2
GAPDH	5'-ATGGTGGTGAAGACGCCAGTA-3'	5'-GGCACAGTCAAGGCTGAGAATG-3'	NM_017008

transcription factors at various stages of osteoblast development (14). Two transcription factors, Runx2 and Osterix, are essential for osteoblastic differentiation during both intramembranous and endochondral bone formation (15-17). Alterations in the function of various non-bone-specific transcription factors such as Msx2 and Dlx5 have also been demonstrated to affect osteoblastic differentiation (18-21). However, the novel zinc finger transcription factor AJ18 has been reported to regulate osteoblastic differentiation (22), but the effect of BA on osteoblastic differentiation through osteogenesis-related transcription factors was not examined. Therefore, in the present study, the effect of BA on osteoblastic differentiation through osteogenesis-related transcription factors, such as Runx2, Osterix, Msx2, Dlx5, and AJ18, was examined using ROS17/2.8 cells as osteoblasts. Additionally, the effect of BA on mineralized nodule formation through ALPase activity and the expression of extracellular matrix proteins such as BSP and osteocalcin was also examined using ROS17/2.8 cells.

Materials and Methods

Cell culture

The rat clonal cell line ROS17/2.8 (23) was employed as osteoblasts. The cells were maintained in α-minimal essential medium (α-MEM; Gibco-BRL, Rockville, MD, USA) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT, USA) and 1% (v/v) penicillin-streptomycin solution (Sigma Chemical, St. Louis, MO, USA) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. For experimental treatments, the cells were seeded into 6-well tissue culture plates at a density of 2×10^4 cells/cm^2 and left overnight to settle. The cells were then cultured in α-MEM with 0, 10^{-5}, 10^{-4}, or 10^{-3} M BA (Wako Pure Chemicals, Osaka, Japan) for up to 7 days. The concentrations of BA were chosen based on previous studies (1,9,13,24).

Real-time PCR

Total RNA was isolated from cultured cells at the indicated time-points using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The mRNA was converted into complementary DNA (cDNA) using an RNA PCR kit (PrimScript; Takara Shuzo, Osaka, Japan). The resulting cDNA mixture was diluted 1:5 in sterile, distilled water, and 2 µl of the diluted cDNA was subjected to real-time PCR analysis using the SYBR Green I dye. The reactions were performed in 25 µl of a SYBR® premixed Ex *Taq*™ solution (Takara Shuzo) containing 20 µM sense and antisense primers (Table 1). The primers were designed using Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA). The PCR assays were performed on a Smart Cycler (Cepheid, Sunnyvale, CA, USA) and analyzed using the instrument's software. The PCR protocol for Runx2, Osterix, Msx2, Dlx5, AJ18, BSP, and osteocalcin consisted of 35 cycles at 95°C for 5 s and 60°C for 20 s. All real-time PCR experiments were performed in triplicate, and the specificity of the PCR products was verified through melting curve analysis. Calculated gene expression levels were normalized to the levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA at the same time.

Mineralized nodule formation

The cells were seeded into 24-well tissue culture plates at a density of 2×10^4 cells/cm^2 and cultured in α-MEM with 50 mM β-glycerophosphate and 50 µg/ml ascorbic acid in the presence or absence of 10^{-5}, 10^{-4}, or 10^{-3} M BA for 7 days. The culture medium was changed every second or third day. The condition of the cells and nodule formation were checked routinely by phase-contrast microscopy (Nikon, Tokyo, Japan). Mineralized nodules were detected by staining with alizarin red (Wako Pure Chemicals), as described previously (13,25).

ALPase activity

The cells were seeded into 96-well microplates at a

density of 2×10^4 cells/cm^2, and cultured in α-MEM with 0, 10^{-5}, 10^{-4}, or 10^{-3} M BA for up to 7 days. Two hundred microliters of enzyme assay solution [8 mM *p*-nitrophenyl phosphate, 12 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 0.1 M glycine-NaOH buffer (pH 10.5)] were added to the cells in each well, and the plate was incubated for several minutes at 37°C. The enzyme reaction was terminated by adding 50 µl of 0.2 M NaOH. The amount of *p*-nitrophenol released by the enzymatic reaction was determined by measuring the absorbance at 405 nm in a microtiter plate reader. One unit of ALPase activity was defined as the amount required for the liberation of 1.0 µmol *p*-nitrophenol per minute. The enzyme activity was recorded as milliunits (mU)/10^4 cells.

SDS-PAGE and Western blotting

The cells were placed in 6-well tissue culture plates at a density of 2×10^4 cells/cm^2 and cultured in α-MEM containing 10% FBS with or without 10^{-3} M BA for 3 days. The culture medium was changed to serum-free α-MEM, and the cells were then cultured for 24 h at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The culture medium containing extracellular matrix proteins was collected and then dialyzed using a PD-10 column (Amersham Biosciences, Piscataway, NJ, USA). The culture medium was subjected to 4-20% gradient SDS-PAGE (8.3 cm × 6.5 cm × 0.75 mm) with a discontinuous Tris-glycine buffer system (26). Samples containing 20 µg of extracellular matrix proteins were dissolved in 10 µl of sample buffer containing 1% SDS, 2 M urea, 15 mg/ml dithiothreitol, and bromophenol blue, and then heated at 95°C for 5 min before loading. Gels were run at 150 V for 60 min.

Gel-separated proteins were transferred to a membrane using a semidry electrotransfer unit with a continuous buffer system consisting of 39 mM glycine, 48 mM Tris, 0.0375% SDS, and 20% (v/v) methanol at 0.8 mA/cm^2 (constant amperage) for 60-90 min. On completion of the transfer, the membrane was treated with 25% (v/v) blocking reagent in Tris-buffered saline (TBS: 10 mM Tris, 145 mM NaCl, pH 7.4) at 4°C for 18 h. The membrane was washed in TBS containing Tween 20 (TBS-Tween) and then incubated at room temperature for 90 min with polyclonal IgG antibodies; i.e., rabbit anti-BSP (Millipore, Billerica, MA, USA), goat anti-osteocalcin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or mouse anti-β-tubulin (Santa Cruz Biotechnology), each diluted 1:500 in 10% (v/v) blocking reagent. The membranes were washed in TBS-Tween and incubated at room temperature for 60 min with the appropriate biotin-conjugated secondary antibodies (1:10,000 in 10% blocking agent): goat anti-rabbit IgG (American Qualex, San Clemente, CA, USA), donkey anti-goat IgG (Chemicon International, Temecula, CA, USA), or goat anti-mouse IgG (Zymed, San Francisco, CA, USA). The membranes were then washed in TBS-Tween and phosphate-buffered saline (PBS; Nissui Pharmaceutical, Tokyo, Japan) and incubated for 30 min at room temperature with horseradish peroxidase-conjugated streptavidin diluted with PBS. Immunoreactive proteins were visualized using a commercial chemiluminescence kit (Amersham Life Sciences, Little Chalfont, Buckinghamshire, UK) and exposed to X-ray film (Eastman Kodak, New Haven, CT, USA). As a control, the membranes were incubated in diluted normal rabbit, goat, or mouse serum, respectively; the dilution factor was the same as that used for the primary antibodies. Pre-stained molecular weight standards run on the same gel were used to estimate protein size.

Statistical analysis

Each value represents the mean ± standard deviation (SD). Significant differences were determined using Bonferroni's modification of Student's *t*-test or an analysis of variance. Differences at $P < 0.05$ were considered statistically significant.

Results

Gene expression of transcription factors

ROS17/2.8 cells were cultured with 0, 10^{-5}, 10^{-4}, or 10^{-3} M BA for up to 7 days, and the gene expression levels of Runx2, Osterix, Dlx5, Msx2, and AJ18 on days 3, 5, and 7 of culture were determined using real-time PCR to estimate the mRNA levels (Fig. 1).

The gene expression levels of Runx2, Osterix, Dlx5, and Msx2 decreased significantly (0.6-0.8-fold) in the presence of 10^{-3} M BA compared to the control on day 3 of culture; the levels of Runx2, Osterix, Dlx5, and Msx2 were not affected by the presence of BA on day 5 (Fig. 1A-D) and the levels for Osterix and Msx2 decreased slightly in the presence of 10^{-4} M BA on day 7 (Fig. 1B and D). However, the gene expression level of AJ18 increased significantly (1.7-fold) in the presence of 10^{-3} M BA compared to the control on days 3 and 5 of culture; the level was not affected by the presence of BA on day 7 (Fig. 1E).

Mineralized nodule formation

ROS17/2.8 cells were cultured with 0, 10^{-5}, 10^{-4}, or 10^{-3} M BA for up to 7 days. Mineralized nodule formation was examined by alizarin red staining on days 5 and 7 of culture (Fig. 2). The intensity of alizarin red staining for

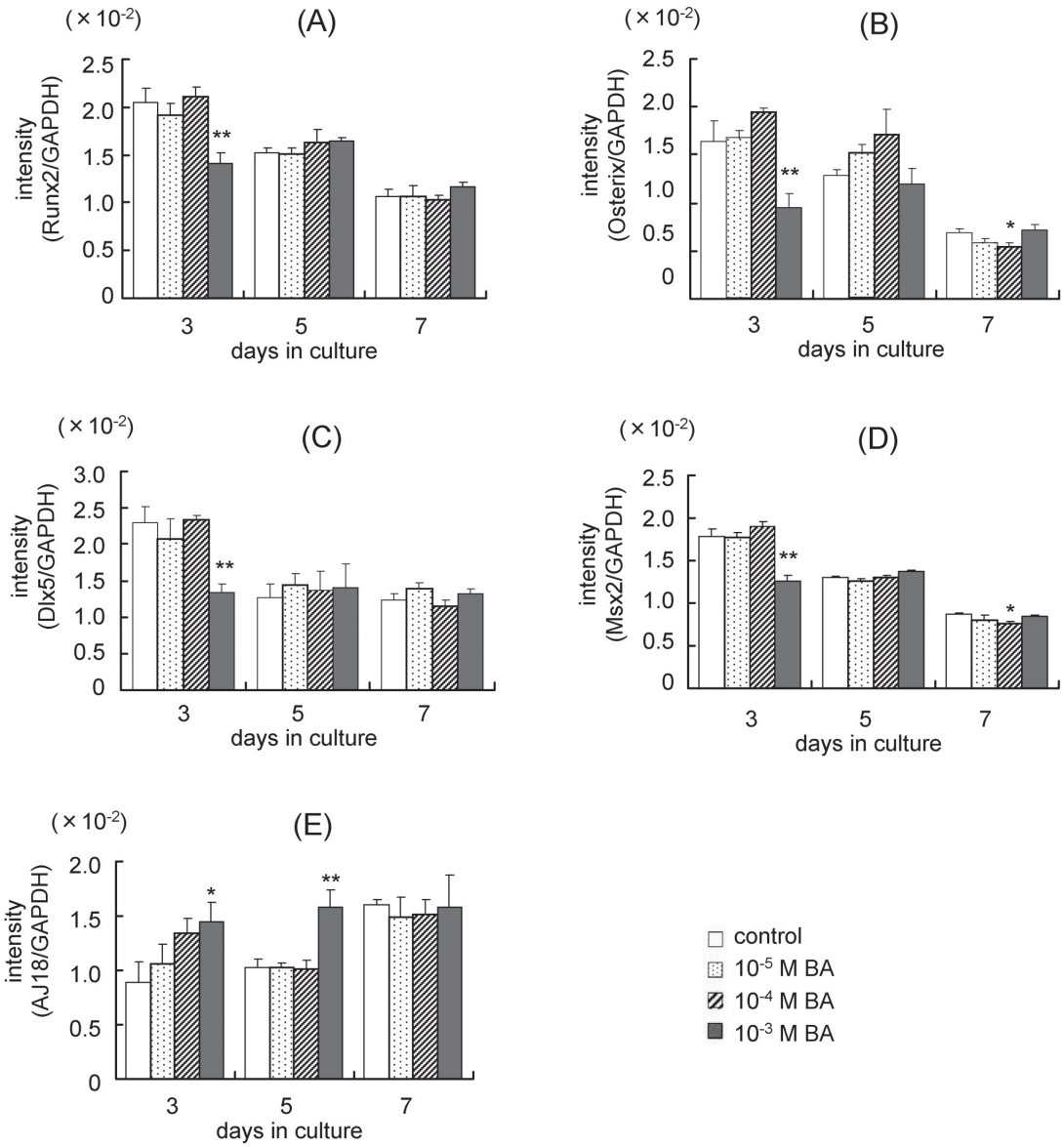

Fig. 1 Effects of butyric acid (BA) on transcription factor gene expression levels. ROS17/2.8 cells were cultured with 0 (control), 10^{-5}, 10^{-4}, or 10^{-3} M BA for up to 7 days, and the gene expression levels of Runx2 (A), Osterix (B), Dlx5 (C), Msx2 (D), and AJ18 (E) on days 3, 5, and 7 of culture were determined using real-time PCR. Each bar indicates the mean ± SD of three separate experiments. *$P < 0.05$, **$P < 0.01$, BA treatment vs. control.

mineralized nodules on day 7 of culture was not affected by the presence of 10^{-5} BA compared to the control, whereas it decreased slightly in the presence of 10^{-4} M BA and was not detected in the presence of 10^{-3} M BA. In contrast, mineralized nodules were detected slightly on day 5 of culture in the absence of BA but were not detected in the presence of BA.

ALPase activity

ROS17/2.8 cells were cultured with 0, 10^{-5}, 10^{-4}, or 10^{-3} M BA for up to 7 days, and ALPase activity was determined on days 3, 5, and 7 of culture (Fig. 3). ALPase activity was not affected by the presence of 10^{-5} and 10^{-4} M BA compared to the control through day 7 of culture. However, the activity decreased significantly (0.6-0.7-fold) in the presence of 10^{-3} M BA compared to the control.

Gene and protein expression of extracellular matrix proteins

ROS17/2.8 cells were cultured with 0, 10^{-5}, 10^{-4}, or 10^{-3} M BA for up to 7 days, and the gene expression levels of

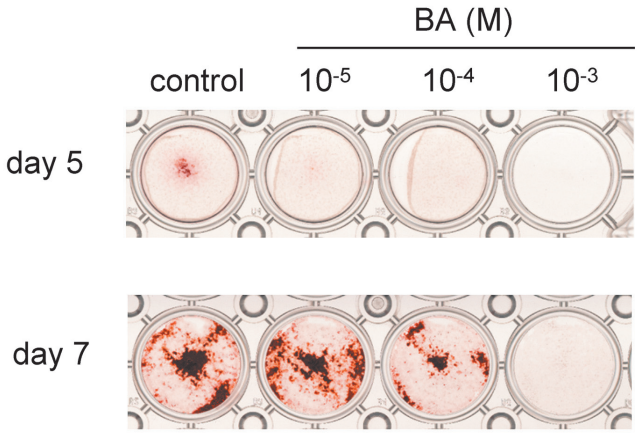

Fig. 2 Effects of butyric acid (BA) on mineralized nodule formation. ROS17/2.8 cells were cultured with 0 (control), 10^{-5}, 10^{-4}, or 10^{-3} M BA for up to 7 days. Mineralized nodules were stained with alizarin red on days 5 and 7 of culture.

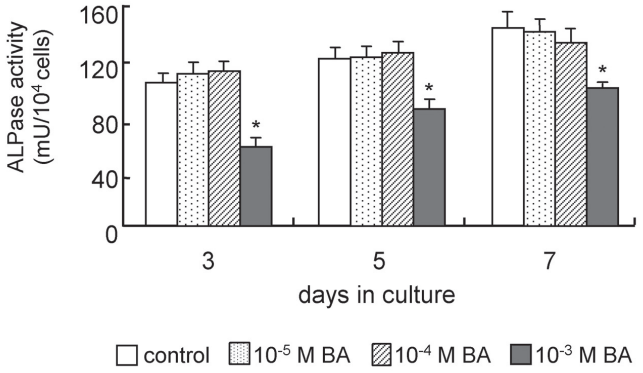

Fig. 3 Effects of butyric acid (BA) on alkaline phosphatase (ALPase) activity. ROS17/2.8 cells were cultured with 0 (control), 10^{-5}, 10^{-4}, or 10^{-3} M BA for up to 7 days, and ALPase activity was determined on days 3, 5, and 7 of culture. Each bar indicates the mean ± SD of three separate experiments. *$P < 0.05$, BA treatment vs. control.

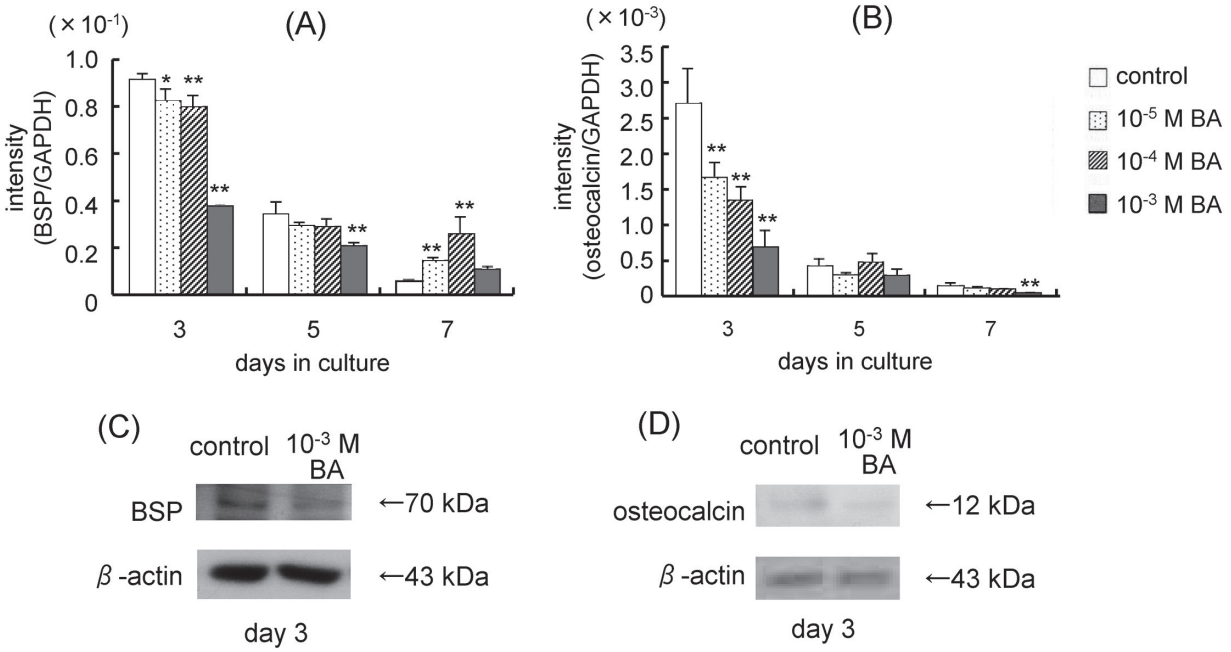

Fig. 4 Effects of butyric acid (BA) on the gene and protein expression levels of extracellular matrix proteins. ROS17/2.8 cells were cultured with 0 (control), 10^{-5}, 10^{-4}, or 10^{-3} M BA for up to 7 days, and the gene expression levels of BSP (A) and osteocalcin (B) on days 3, 5, and 7 of culture were determined using real-time PCR. The protein expression levels of BSP (C) and osteocalcin (D) on day 3 of culture were examined by Western blotting. Each bar indicates the mean ± SD of three separate experiments. *$P < 0.05$, **$P < 0.01$, BA treatment vs. control.

BSP and osteocalcin on days 3, 5, and 7 of culture were determined using real-time PCR to estimate the mRNA levels (Fig. 4A and B). The effects of 10^{-3} M BA on the protein expression levels of BSP and osteocalcin on day 3 of culture were examined by Western blotting (Fig. 4C and D).

The gene expression levels of BSP and osteocalcin decreased markedly (0.4- and 0.2-fold, respectively) in the presence of 10^{-3} M BA compared to the control on day 3 of culture: 0.9- and 0.6-fold, respectively, in the

presence of 10^{-4} M BA; 0.9- and 0.6-fold, respectively, in the presence of 10^{-5} M BA. The BSP and osteocalcin protein expression levels decreased in the presence of 10^{-3} M BA compared to the control as well as gene expression on day 3 of culture. The BSP gene expression level decreased slightly (0.8-fold) in the presence of 10^{-3} M BA on day 5 of culture, whereas osteocalcin expression was not affected by the presence of BA. The BSP gene expression levels increased significantly (4.0-fold) in the presence of 10^{-4} M BA compared to the control on day 7 of culture, whereas osteocalcin expression decreased significantly (0.5-fold) in the presence of 10^{-3} M BA.

Discussion

It has been hypothesized that BA infiltrated the gingival epithelium and influence the differentiation and function of osteoblasts in alveolar bone. To demonstrate this hypothesis, the effect of BA on osteoblastic differentiation through the expression of osteogenesis-related transcription factors was examined using ROS17/2.8 cells as osteoblasts. Additionally, the effect of BA on mineralized nodule formation through ALPase activity and the expression of extracellular matrix proteins were also examined using ROS17/2.8 cells.

In the present study, the expression of Runx2, Osterix, Dlx5, and Msx2 in ROS17/2.8 cells decreased significantly in the presence of 10^{-3} M BA compared to the control, whereas AJ18 expression increased significantly. Runx2 is essential for osteoblast differentiation of mesenchymal stem cells. Furthermore, mesenchymal stem cells differentiate into mature osteoblasts, which express high levels of osteocalcin (27). In the present study, not only Runx2 expression, but also osteocalcin expression in ROS17/2.8 cells, decreased in the presence of 10^{-3} M BA. Runx2 is also essential for chondrocyte maturation, which is a prerequisite for endochondral ossification (27). Osterix, which has a three zinc-finger motif, is a second transcription factor essential for osteoblast differentiation (17). Two homeobox transcription factors, Dlx5 and Msx2, appear to regulate the development of mineralized tissues, including bone, cartilage, and teeth (27). Dlx5 expression is correlated with osteoblast differentiation, and maximal Dlx5 expression occurs during the final stages of *in vitro* osteoblast differentiation, suggesting that Dlx5 may be involved in the maturation of the bone cell phenotype (28).

The function of Msx2 in osteoblasts is still controversial. Liu et al. (19) suggested that Msx2 inhibits differentiation of osteoblast precursors and immature osteoblasts, resulting in an increase in the source of osteoblastic cells. However, other studies have demonstrated that Msx2 promotes osteoblast differentiation and/or proliferation (29,30). From the results in the present study, the function of Msx2 is considered to promote osteoblastic differentiation. AJ18 may downregulate osteoblast differentiation by binding to osteoblast-specific element 2 and modulate transactivation by Runx2 (22). In light of these findings, the results of the present study suggest that 10^{-3} M BA suppressed osteoblastic differentiation by decreasing Runx2, Osterix, Dlx5, and Msx2 expression and by increasing AJ18 expression.

ALPase, which hydrolyzes the ester bond of organic phosphate compounds under alkaline conditions, plays an important role in bone calcification. Not only does the enzyme hydrolyze substances that inhibit calcification, such as pyrophosphate and ATP, it is also indispensable for producing the increased phosphate concentration required for hydroxyapatite crystallization (31). In contrast, non-collagenous matrix proteins such as BSP and osteocalcin are believed to be important in the organization of the collagen matrix and in regulating the formation and growth of hydroxyapatite crystals (32). Katono et al. (13) reported that 10^{-4} M BA stimulates mineralized nodule formation by increasing BSP and osteopontin production in human osteoblasts on day 10 of culture, whereas in the present study, ALPase activity was not affected by presence of 10^{-4} M BA on days 3, 5, and 7 of culture. Furthermore, Young et al. (33) reported that 10^{-4} M BA stimulates BSP gene transcription in ROS17/2.8 cells at 12 h.

Therefore, the effects of BA on mineralized nodule formation, ALPase activity, and the expression of extracellular matrix proteins using ROS17/2.8 cells were examined. In the present study, mineralized nodule formation was suppressed slightly in the presence of 10^{-4} M BA, and it was suppressed markedly in the presence of 10^{-3} M BA. Differences in cell type and BA concentration might be causes of the discrepancy between the present results and those of Katono et al. (13). ALPase activity was not affected by the presence of 10^{-4} M BA until day 7 of culture in this study, as well as in the study by Katono et al. (13). However, activity decreased significantly in the presence of 10^{-3} M BA on days 3, 5, and 7 of culture; Katono et al. (13) did not show the effect of 10^{-3} M BA on ALPase activity. Furthermore, BSP and osteocalcin expression decreased significantly in the presence of 10^{-3} M BA on days 3, 5, and/or 7 of culture. BSP expression increased significantly in the presence of 10^{-4} M BA on day 7 of culture in this study, as well as in the study by Katono et al. (13) in which they did not show the effects of 10^{-3} M BA on BSP expression. The effect of BA on BSP expression in the present study might be different from

the report by Yang et al. (33). However, the concentration and time of stimulation by BA was obviously different in these studies; i.e., 10^{-4} M BA and 12 h in Yang et al. (33) and 10^{-3} M BA and 3 or 5 days in this study. It is difficult to compare the results that have been reported in previous publications. However, the findings in this study have important implications for understanding osteoblastic differentiation and mineralized nodule formation. High concentration of BA suppresses mineralized nodule formation by decreasing ALPase activity and the expression of BSP and osteocalcin.

In conclusion, the results suggest that 10^{-3} M BA suppressed osteoblastic differentiation by decreasing Runx2, Osterix, Dlx5, and Msx2 expression and by increasing AJ18 expression. Moreover, BA suppressed bone formation by decreasing ALPase activity and the expression of BSP and osteocalcin. Furthermore, the current findings suggest that the elevated concentration of BA, which is a major metabolic by-product of anaerobic Gram-negative periodontopathic bacteria in subgingival plaque, caused a decrease in bone formation during alveolar bone remodeling.

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References

1. Niederman R, Buyle-Bodin Y, Lu BY, Robinson P, Naleway C (1997) Short-chain carboxylic acid concentration in human gingival crevicular fluid. J Dent Res 76, 575-579.
2. Tonetti M, Eftimiadi C, Damiani G, Buffa P, Buffa D, Botta GA (1987) Short chain fatty acids present in periodontal pockets may play a role in human periodontal diseases. J Periodontal Res 22, 190-191.
3. Singer RE, Buckner BA (1981) Butyrate and propionate: important components of toxic dental plaque extracts. Infect Immun 32, 458-463.
4. Jeng JH, Chan CP, Ho YS, Lan WH, Hsieh CC, Chang MC (1999) Effects of butyrate and propionate on the adhesion, growth, cell cycle kinetics, and protein synthesis of cultured human gingival fibroblasts. J Periodontol 70, 1435-1442.
5. Kurita-Ochiai T, Hashizume T, Yonezawa H, Ochiai K, Yamamoto M (2006) Characterization of the effects of butyric acid on cell proliferation, cell cycle distribution and apoptosis. FEMS Immunol Med Microbiol 47, 67-74.
6. Kurita-Ochiai T, Fukushima K, Ochiai K (1997) Butyric acid-induced apoptosis of murine thymocytes, splenic T cells, and human Jurkat T cells. Infect Immun 65, 34-41.
7. Kurita-Ochiai T, Ochiai K, Fukushima K (2000) Butyric-acid-induced apoptosis in murine thymocytes and splenic T- and B-cells occurs in the absence of p53. J Dent Res 79, 1948-1954.
8. Kurita-Ochiai T, Ochiai K, Fukushima K (2001) Butyric acid-induced T-cell apoptosis is mediated by caspase-8 and -9 activation in a Fas-independent manner. Clin Diagn Lab Immunol 8, 325-332.
9. Kurita-Ochiai T, Ochiai K, Suzuki N, Otsuka K, Fukushima K (2002) Human gingival fibroblasts rescue butyric acid-induced T-cell apoptosis. Infect Immun 70, 2361-2367.
10. Kurita-Ochiai T, Amano S, Fukushima K, Ochiai K (2003) Cellular events involved in butyric acid-induced T cell apoptosis. J Immunol 171, 3576-3581.
11. Kurita-Ochiai T, Seto S, Ochiai K (2004) Role of cell-cell communication in inhibiting butyric acid-induced T-cell apoptosis. Infect Immun 72, 5947-5954.
12. Schroeder TM, Westendorf JJ (2005) Histone deacetylase inhibitors promote osteoblast maturation. J Bone Miner Res 20, 2254-2263.
13. Katono T, Kawato T, Tanabe N, Suzuki N, Iida T, Morozumi A, Ochiai K, Maeno M (2008) Sodium butyrate stimulates mineralized nodule formation and osteoprotegerin expression by human osteoblasts. Arch Oral Biol 53, 903-909.
14. Yang X, Karsenty G (2002) Transcription factors in bone: developmental and pathological aspects. Trends Mol Med 8, 340-345.
15. Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, Stamp GW, Beddington RS, Mundlos S, Olsen BR, Selby PB, Owen MJ (1997) Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell 89, 765-771.
16. Karsenty G (2000) Role of Cbfa1 in osteoblast differentiation and function. Semin Cell Dev Biol 11, 343-346.

17. Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrugghe B (2002) The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 108, 17-29.
18. Acampora D, Merlo GR, Paleari L, Zerega B, Postiglione MP, Mantero S, Bober E, Barbieri O, Simeone A, Levi G (1999) Craniofacial, vestibular and bone defects in mice lacking the Distal-less-related gene Dlx5. Development 126, 3795-3809.
19. Liu YH, Tang Z, Kundu RK, Wu L, Luo W, Zhu D, Sangiorgi F, Snead ML, Maxon RE (1999) Msx2 gene dosage influences the number of proliferative osteogenic cells in growth centers of the developing murine skull: a possible mechanism for MSX2-mediated craniosynostosis in humans. Dev Biol 205, 260-274.
20. Miyama K, Yamada G, Yamamoto TS, Takagi C, Miyado K, Sakai M, Ueno N, Shibuya H (1999) A BMP-inducible gene, Dlx5, regulates osteoblast differentiation and mesoderm induction. Dev Biol 208, 123-133.
21. Satokata I, Ma L, Ohshima H, Bei M, Woo I, Nishizawa K, Maeda T, Takano Y, Uchiyama M, Heaney S, Peters H, Tang Z, Maxson R, Maas R (2000) Msx2 deficiency in mice causes pleiotropic defects in bone growth and ectodermal organ formation. Nat Genet 24, 391-395.
22. Jheon AH, Ganss B, Cheifetz S, Sodek J (2001) Characterization of a novel KRAB/C_2H_2 zinc finger transcription factor involved in bone development. J Biol Chem 276, 18282-18289.
23. Majeska RJ, Nair BC, Rodan GA (1985) Glucocorticoid regulation of alkaline phosphatase in the osteoblastic osteosarcoma cell line ROS17/2.8. Endocrinology 116, 170-179.
24. Iida T, Kawato T, Tanaka H, Tanabe N, Nakai K, Zhao N, Suzuki N, Ochiai K, Maeno M (2011) Sodium butyrate induces the production of cyclo-oxygenases and prostaglandin E_2 in ROS 17/2.8 osteoblastic cells. Arch Oral Biol 56, 678-686.
25. Williams DC, Boder GB, Toomey RE, Paul DC, Hillman CC Jr, King KL, Van Frank RM, Johnston CC Jr (1980) Mineralization and metabolic response in serially passaged adult rat bone cells. Calcif Tissue Int 30, 233-246.
26. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.
27. Komori T (2006) Regulation of osteoblast differentiation by transcription factors. J Cell Biochem 99, 1233-1239.
28. Hassan MQ, Javed A, Morasso MI, Karlin J, Montecino M, van Wijnen AJ, Stein GS, Stein JL, Lian JB (2004) Dlx3 transcriptional regulation of osteoblast differentiation: temporal recruitment of Msx2, Dlx3, and Dlx5 homeodomain proteins to chromatin of the osteocalcin gene. Mol Cell Biol 24, 9248-9261.
29. Ishii M, Merrill AE, Chan YS, Gitelman I, Rice DP, Sucov HM, Maxson RE Jr (2003) Msx2 and Twist cooperatively control the development of the neural crest-derived skeletogenic mesenchyme of the murine skull vault. Development 130, 6131-6142.
30. Ichida F, Nishimura R, Hata K, Matsubara T, Ikeda F, Hisada K, Yatani H, Cao X, Komori T, Yamaguchi A, Yoneda T (2004) Reciprocal roles of Msx2 in regulation of osteoblast and adipocyte differentiation. J Biol Chem 279, 34015-34022.
31. Anderson HC (1989) Mechanism of mineral formation in bone. Lab Invest 60, 320-330.
32. Ganss B, Kim RH, Sodek J (1999) Bone sialoprotein. Crit Rev Oral Biol Med 10, 79-98.
33. Young L, Li Z, Li X, Wang Z, Wang S, Sasaki Y, Takai H, Ogata Y (2010) Butyric acid stimulates bone sialoprotein gene transcription. J Oral Sci 52, 231-237.