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Butyrate induces reactive oxygen species production and affects cell cycle progression in human gingival fibroblasts

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Background and Objective: Short-chain fatty acids, such as butyric acid and propionic acid, are metabolic by-products generated by periodontal microflora such as *Porphyromonas gingivalis*, and contribute to the pathogenesis of periodontitis. However, the effects of butyrate on the biological activities of gingival fibroblasts (GFs) are not well elucidated.

Material and Methods: Human GFs were exposed to various concentrations of butyrate (0.5–16 mM) for 24 h. Viable cells that excluded trypan blue were counted. Cell cycle distribution of GFs was analyzed by propidium iodide-staining flow cytometry. Cellular reactive oxygen species (ROS) production was measured by flow cytometry using 2',7'-dichlorofluorescein (DCF). Total RNA and protein lysates were isolated and subjected to RT-PCR using specific primers or to western blotting using specific antibodies, respectively.

Results: Butyrate inhibited the growth of GFs, as indicated by a decrease in the number of viable cells. This event was associated with an induction of G0/G1 and G2/M cell cycle arrest by butyrate (4–16 mM) in GFs. However, no marked apoptosis of GFs was noted in this experimental condition. Butyrate (> 2 mM) inhibited the expression of *cdc2*, *cdc25C* and cyclinB1 mRNAs and reduced the levels of Cdc2, Cdc25C and cyclinB1 proteins in GFs, as determined using RT-PCR and western blotting, respectively. This toxic effect of butyrate was associated with the production of ROS.

Conclusion: These results suggest that butyrate generated by periodontal pathogens may be involved in the pathogenesis of periodontal diseases via the induction of ROS production and the impairment of cell growth, cell cycle progression and expression of cell cycle-related genes in GFs. These events are important in the initiation and prolongation of inflammatory processes in periodontal diseases.

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Periodontal pathogens may generate various virulence factors, such as proteases, endotoxin, peptidoglycans and organic acids, to promote the onset and progression of periodontal disease (1), and some periodontal pathogens, such as *Eubacterium, Fusobacteria* and *Peptococci*, may use amino acids, hexose or pentose to generate differential

amounts of butyric acid in periodontal pockets (2,3). Short-chain fatty acids (SCFAs), such as acetic acid, propionic acid and butyric acid, are important in the initiation and propagation of periodontal diseases (1,3); they are generally present at millimolar concentrations in the gingival crevicular fluid from periodontal pockets of diseased sites, and their concentrations decrease after scaling and root planing (3,4). The levels of SCFAs in gingival crevicular fluid were also reported to be associated with the severity of periodontal diseases (5). The mean concentration of butyric acid in gingival crevicular fluid collected from sites with severe periodontitis is about 2.6 mM, in contrast to 0.2 mM at sites with mild periodontitis and undetectable in healthy sites (5), or 0.5–16 mM at sites with different disease status (6).

At higher concentrations, butyrate may suppress the proliferation of endothelial cells, gingival epithelial cells and fibroblasts. It also inhibits leukocyte apoptosis and function, but stimulates the release of cytokines from leukocytes (3,7). Several studies report that butyrate suppresses the adhesion, protein synthesis as well as cell cycle progression of GF and SAS tongue cancer epithelial cells (6–8). Butyric acid also significantly inhibits the proliferation and the concanavalin A-stimulated production of interleukin (IL)-2, IL-4, IL-5, IL-6 and IL-10 in splenic T cells (9). Direct application of butyric acid to healthy human gingiva may elicit cytokine release and a tissue inflammatory response (3). All these events may contribute to tissue damage and to periodontal pocket formation.

Reactive oxygen species (ROS) have been shown to be crucial for inducing genetic and epigenetic alterations of cells and contribute to chemical toxicity as well as to carcinogenesis (10,11). A recent report demonstrates that ROS levels are related to bony destruction in periodontitis (12). Gingival fibroblasts (GFs) cultured from healthy gingiva are more resistant to butyric acid-induced apoptosis than are GFs cultured from inflamed gingiva, in which marked apoptosis is induced by butyrate (13). However, the reasons why exposure of GFs to high concentrations of SCFAs in gingival crevices may lead to tissue destruction and pocket formation should be further addressed. Butyrate has been shown to inhibit the growth of gingival cells and potentially to contribute to periodontal inflammation. Cell growth is critically regulated by the cell cycle and by cell cycle-related genes such as *cdc2*, *cdc25* and cyclins (14,15). We proposed that SCFAs, such as butyrate, may impair periodontal tissue healing via inhibition of cell growth and cell cycle progression of GFs, and contribute to the pathogenesis of periodontitis. ROS may be involved in these events.

Material and methods

Materials

All cell culture materials were purchased from Gibco Laboratories (Life Technologies, Grand Island, NY, USA). Propidium iodide (PI), sodium butyrate and 2',7'-dichlorodihydrofluorescein diacetate were purchased from Sigma (Sigma Chemical Company, St Louis, MO, USA). PCR primers were synthesized by Genemed Biotechnologies, Inc. (San Francisco, CA, USA). Mouse anti-bodies against human cyclin B1, Cdc2, Cdc25C and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Igs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Flow cytometric analysis reagents were from Becton-Dickinson (San Jose, CA, USA).

Culture of human GFs

Following approval of the Ethics Committees of National Taiwan University Hospital or Chang Gung Memorial Hospital, healthy gingival tissues were obtained during crown-lengthening procedures from patients after obtaining their informed consent. Gingival tissues were cut into small pieces using a surgical knife, and GFs were cultured using a tissue-explant technique in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1 × penicillin/100 μg/mL of streptomycin, as described previously (8,16,17). Cells at passages 3–10 were used in these studies. Three GF strains were established and utilized in these studies, with similar results obtained for each.

Effect of butyrate on the growth and cell cycle progression of GFs

In brief, 2.5×10^5 GFs were inoculated onto six-well culture dishes. After 24 h of cell attachment, they were exposed to different concentrations of butyrate (1–16 mM) for 24 h. The same volume of filtered double-distilled water was used as the control. After 24 h, floating cells in the culture medium were collected. Attached GFs were detached from the wells of the culture by trypsin/EDTA. Then, both floating cells and attached cells were collected together. We directly counted, by microscopy, the total number of viable cells that excluded trypan blue dye, as described previously (18).

For further cell cycle analysis (19,20), the collected cells were washed with phosphate-buffered saline (PBS), fixed for 30 min in 70% ice-cold ethanol containing 2 mg/mL of RNase and then stained for 10 min with 40 μg/mL of PI. The fluorescence of PI in GFs was determined by flow cytometric analysis (FACSCalibur; Becton Dickinson, Worldwide Inc., San Jose, CA, USA). The wavelength of laser excitation was set at 488 nm with an emission wavelength collected at higher than 590 nm. The PI fluorescence of 20,000 cells was analyzed for both control and experimental samples. We counted the percentage of cells in sub-G0/G1, G0/G1-, S- and G2/M phases by using MODIFIT software and CELLQUEST programs (Becton Dickinson) (7,19,20).

To evaluate the effect of butyrate on the cell cycle progression of GFs in the exponential growth phase, cells were plated at 1.5×10^5 GFs per well of a six-well plate and exposed to butyrate (4 and 16 mM) for 6, 24 and 48 h. Cells were then collected and the cell cycle distribution of GFs was studied by PI flow cytometry, as described above.

Effect of butyrate on apoptosis – PI/annexin V dual-fluorescence flow cytometry

GFs (2.5×10^5 cells/well) were exposed to various concentrations of

butyrate for 24 h. Then, both floating and attached cells were harvested and the cells were washed with PBS and suspended in 400 μL of HEPES (10 mM HEPES–NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) solution. Then, anti-annexin V–fluorescein isothiocyanate (Becton Dickson)/PI (50 μg/mL) staining solution was added to the cells, which were incubated in the dark for 30 min. The annexin V–fluorescein isothiocyanate and PI dual fluorescence of cultured cells were analyzed immediately by FACSCalibur Flow Cytometry (Becton Dickinson) and a total of 15,000 events were counted as described previously (18).

Effects of butyrate on cell cycle-related gene expression

RNA was isolated from GFs, as follows. In brief, 1.5×10^6 GFs were inoculated onto 10-cm culture dishes. After 24 h of cell attachment, the GFs were exposed to different concentrations of butyrate (2–16 mM) for 24 h. Total RNA was isolated using an RNA isolation kit (Qiagen company, Taiwan) (21).

RT-PCR was performed using specific primers for beta-actin, *cdc2, cdc25C* and cyclinB1, as described previously (22). Briefly, 3 μg of denatured total RNA was reverse transcribed in a total volume of 44.5 μL of reaction mixture containing 4 μL of random primer (500 μg/mL), 8 μL of deoxyribonucleotide triphosphate (2.5 mM), 4.5 μL of 10× RT buffer, 1 μL of RNase inhibitor (40 U/μL) and 0.5 μL of reverse transcriptase (21 U/μL), at 42°C for 90 min. Then, we used 4 μL of cDNA for PCR amplification in a reaction volume of 50 μL comprising 5 μL of 10 × Super TAQ buffer, 4 μL of deoxyribonucleotide triphosphate (2.5 mM), 1 μL of each specific primer and 0.2 μL of Super TAQ enzyme (2 U/μL). The PCR reaction was conducted at 94°C for 5 min for the first cycle, and then further amplified for 15–30 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min with a thermal cycler (Perkin Elmer 4800; PE Applied Biosystems, Foster City, CA, USA). Finally, the reaction was terminated at 72°C for a further 10 min. The specific primer pairs for this study were: *cdc2*: GGGGATTCAGAAATTGATCA and TGTCAGAAAGCT ACATCTTC (288 bp); cyclin B1: AAGAGCTTT AAACTTTGGTCT GGG and CT TTGTAAGTCCTTGATTTACCATG (317 bp); *cdc25c*: CCTGGTGAGAA TTCGAAGACC and GCAGATGA AGTACACATTG CATC (456 bp); and beta-actin: AAGAGAGGCAT CCTCACCCT and TACATGGCT GGGGTGTTGAA (218 bp) (8,19,21). The PCR-amplified products were subjected to electrophoresis on a 1.8% agarose gel and then the gels were stained with ethidium bromide and photographed. That showed linear in relation to the input RNA. Amplification of the *bac* gene was used as control.

Effects of butyrate on cell cycle-related protein expression

Briefly, 1.5×10^6 GFs were inoculated onto 10-cm culture dishes. After 24 h of cell attachment they were exposed to different concentrations of butyrate (1–8 mM) for 24 h. After washing with PBS, the cells were disrupted in lysis buffer (10 mM Tris–HCl, pH 7, 140 mM sodium chloride, 3 mM magnesium chloride, 0.5% Nonidet P-40, 2 mM phenylmethanesulfonyl fluoride, 1% aprotinin and 5 mM dithiothreitol) (19,21). The aliquots (containing 20–50 μg of protein) of cell lysate were electrophoresed through a 12.5% sodium dodecyl sulfate–polyacrylamide gel and then transferred to a polyvinylidene fluoride membrane. The membrane was blotted with mouse anti-human Cdc2, Cdc25c, cyclin B1 and GAPDH Igs for 2 h and then incubated for 1 h with goat anti-mouse horseradish peroxidase-conjugated secondary Igs (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). After washing the membrane with buffer, ECL reagents (Amersham, Pharmacia Biotech, Piscataway, NJ, USA) were added and the chemiluminescence was detected by exposure of the membranes to Fuji films for 30 s to 10 min. The intensity of GAPDH was used as control.

Effect of butyrate on cellular ROS levels

Briefly, 2.5×10^5 GFs were seeded into each well of six-well culture dishes. After 24 h of cell adhesion, the GFs were incubated for 24 h in culture medium containing different concentrations of butyrate (1–16 mM). The ROS level in GFs was determined by single-cell 2',
7'-dichlorofluorescein (DCF) fluorescence flow-cytometric analysis, as described previously (18,20). Briefly, GFs were labeled for 30 min with 10 μM 2',7'-dichlorodihydrofluorescein diacetate, washed with PBS, collected and immediately subjected to flow cytometry analysis (Becton Dickinson) (18,20).

Statistical analysis

At least four or more separate experiments were conducted, and similar results were obtained for each. The results were analyzed by one-way ANOVA and the post-hoc Tukey test. A $p < 0.05$ was considered to indicate a statistically significant difference between two groups.

Results

Morphological changes of GFs induced by butyrate

Untreated GFs were spindle-shaped in appearance (Fig. 1A). After 24 h of incubation in 4 and 8 mM butyrate, the morphological changes of GFs were not as evident (Fig. 1B and 1C) as those after 24 h of incubation in 16 mM butyrate, where the GFs were larger and flatter in appearance (arrows). There was no marked increase in the number of floating cells observed by microscopy (Fig. 1D).

Effect of butyrate on the growth of GFs

Exposure of GFs (2.5×10^5 cells per six-well plate) to butyrate for 24 h slightly inhibited their growth, as determined by counting viable cells that excluded trypan blue. As shown

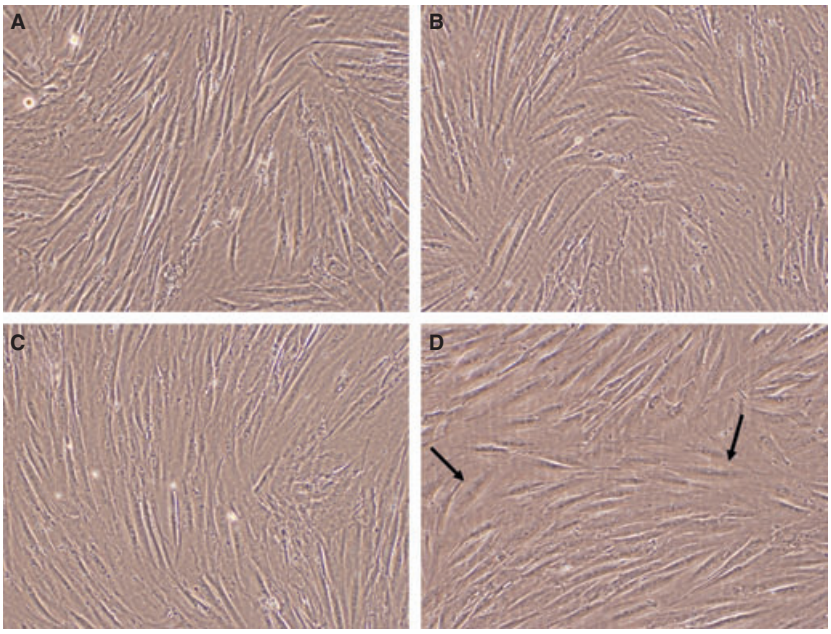

Fig. 1. Morphological changes in gingival fibroblasts (GFs) after exposure to butyrate for 24 h. Control GFs (A) and GFs after exposure to 4 mM (B), 8 mM (C) and 16 mM (D) butyrate. Arrows indicate GFs showing a morphological change (original magnification, 100 ×).

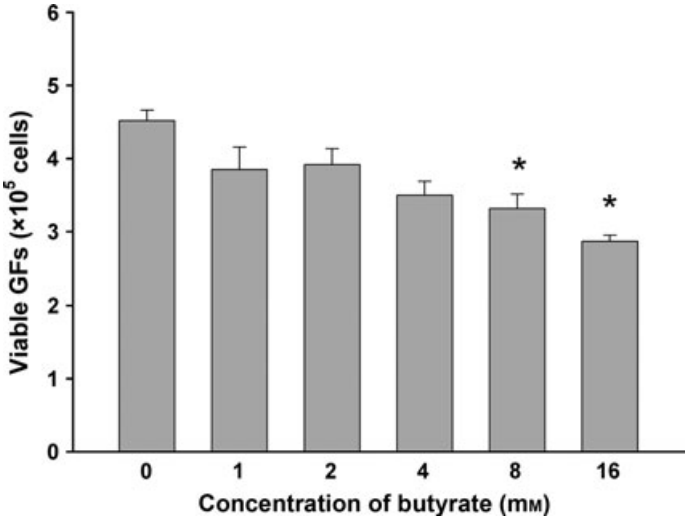

Fig. 2. Effect of butyrate on the growth of gingival fibroblasts (GFs). GFs (2.5×10^5 cells) were exposed to various concentrations of butyrate for 24 h. Cells were then collected and GFs that excluded trypan blue dye were counted. The results are expressed as the number of viable cells ($\times 10^5$ cells, mean ± standard error). *Significant difference compared with the control ($p < 0.05$).

in Fig. 2, about 4.5×10^5 GFs were present in untreated culture. Exposure to 8 and 16 mM butyrate for 24 h resulted in a significant decrease in the number of viable GFs, to 3.3×10^5 and 2.8×10^5 cells, respectively.

Effect of butyrate on cell cycle progression and apoptosis of GFs

Butyrate induced G0/G1 cell cycle arrest in cultured GFs (2.5×10^5 GFs per well) at concentrations of 4 mM. However, at concentrations of 8–16 mM, butyrate induced G2/M cell cycle arrest in GFs ($p > 0.05$) (Fig. 3A). Annexin V/PI dual flow cytometry detected a slight increase in the numbers of apoptotic and necrotic GFs (later apoptotic cells and necrotic cells) after exposure to 16 mM butyrate for 24 h (Fig. 3B). However, this occurred in only a small population of GFs.

When GFs in the exponential phase of growth (1.5×10^5 GFs per well) were exposed to butyrate (4 and 16 mM) for 6, 24 and 48 h, butyrate (4 and 16 mM) induced cell cycle arrest at G2/M and G0/G1, even after only 6 h (Fig. 4). After 48 h of culture, the GFs reached confluence, as shown by a marked increase in the population of cells at G0/G1, from 64% (6 h, control) to 86% (48 h, control). A decrease in the population of GFs at S-phase, from 21.3% (6 h, control) to 12.8% (48 h, control) was also noted, indicating a decrease of cell proliferation 72 h after cell seeding (Fig. 4A and 4C).

Effect of butyrate on cell cycle-related gene expression

A decrease in the expression of *cdc2*, *cdc25C* and cyclinB1 mRNAs was also noted. Butyrate concentrations of > 2 mM decreased the expression of cell cycle-related genes in GFs (Fig. 5).

Effect of butyrate on the levels of cell cycle-related proteins

A decrease in the level of Cdc2 protein was noted at butyrate concentrations higher than 1 mM. The levels of Cdc25C and cyclinB1 proteins also decreased when the butyrate concentration was 2 mM or higher (Fig. 6).

Effect of butyrate on ROS production by GFs

Exposure of GFs to butyrate for 24 h markedly elevated the cellular DCF fluorescence. As indicated in the representative histogram, a shift of cellular DCF fluorescence (FL1-H) to the right was noted (Fig. 7A). Quantitatively, the mean DCF fluorescence of GFs was elevated from 108 (control) to 233 and 287, by 4 and 8 mM butyrate,

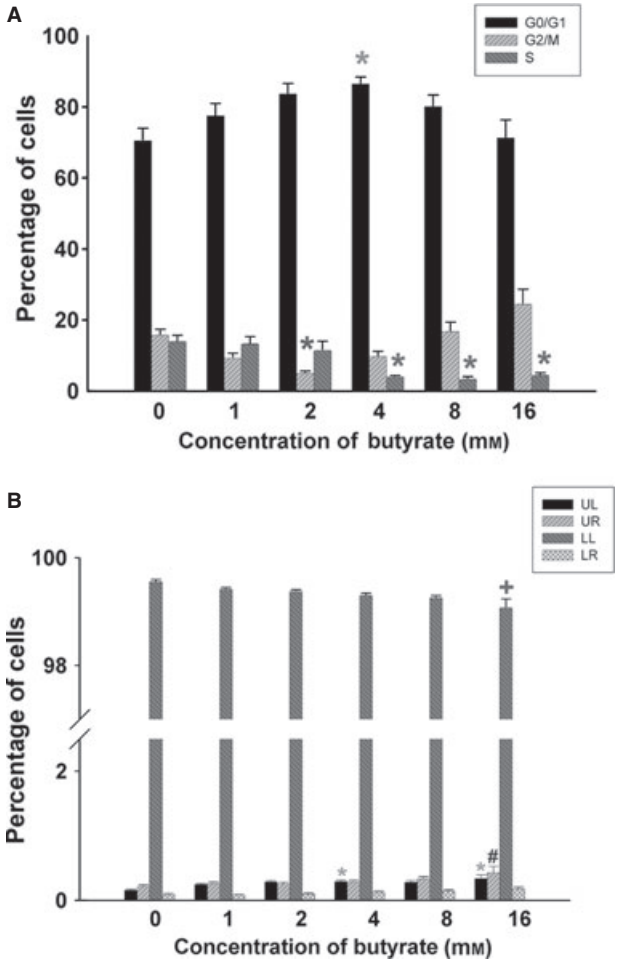

Fig. 3. Effect of butyrate on cell cycle progression and apoptosis of gingival fibroblasts (GFs). GFs (2.5×10^5 cells per well) were exposed to various concentrations of butyrate for 24 h. Both floating cells and attached GFs were collected. Cells were fixed and stained with propidium iodide (PI) and subjected to flow cytometric analysis. (A) Percentage of cells (mean ± standard error) in G0/G1, S and G2/M phases of the cell cycle. *Significant difference compared with the control ($p < 0.05$). (B) The collected GFs were directly stained with PI and annexin V and subjected to flow cytometry analysis. Results were expressed as percentages of necrotic cells (UL), viable cells (LL), later apoptotic cells (UR) and early apoptotic cells (LR). * $p < 0.05$, UL; # $p < 0.05$, UR; + $p < 0.05$, LL; all compared with the control (0 mM butyrate).

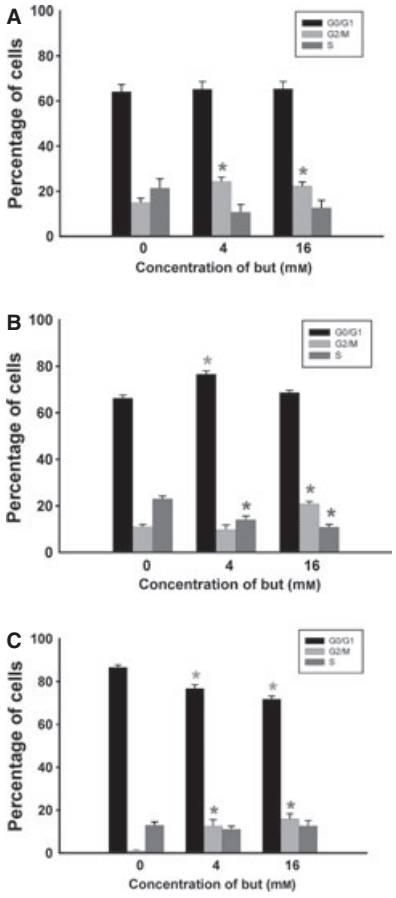

Fig. 4. Effect of butyrate on cell cycle distribution of gingival fibroblasts (GFs) cultured in exponential growth phase. GFs (1.5×10^5 cells/well) were exposed to butyrate (4 and 16 mM) for (A) 6 h, (B) 24 h and (C) 48 h. Cells were collected and cell cycle distribution analysis was performed using propidium iodide (PI) flow cytometry. The results were expressed as the percentage of cells (mean ± standard error) in G0/G1, S and G2/M phases of the cell cycle. *Significant difference compared with the control (0 mM butyrate) ($p < 0.05$).

respectively (Fig. 7B), indicating the induction of ROS production.

Discussion

Butyric acid is a metabolic by-product produced by a number of periodontal pathogens at millimolar concentrations in the gingival crevice. Butyrate has been suggested to inhibit the growth of GFs and epithelial cells (3,7). After 24 h, it induces G0/G1 arrest of GFs when used at concentrations of 2–8 mM, but leads to G2/M arrest when used at concentrations higher than 8 mM (7). Accordingly, in the present study, butyrate inhibited the proliferation of GFs without affecting cell viability, when analyzed using trypan blue dye exclusion. Interestingly, GFs became flattened after exposure to butyrate for 24 h. A similar increase in cell size of GFs after exposure to *Actinobacillus actinomycetemcomitans* has been reported (23). This may be associated with alterations in the GF cytoskeleton; however, the reasons are not fully understood. Gingival epithelium and connective tissue are two barriers that prevent invasion with pathogenic microorganisms and are important for periodontal wound healing. As the butyrate concentration in gingival crevicular fluid is about 0.25–16 mM (6), generation of butyric acid in the gingival sulcus may contribute to the pathogenesis of periodontitis by disruption of periodontal tissue and impairment of periodontal healing.

GFs (1.5×10^5 cells per well of a six-well plate) had reached confluence after 48 h of culture, as shown by an increase of the G0/G1 population. This is generally in accordance with

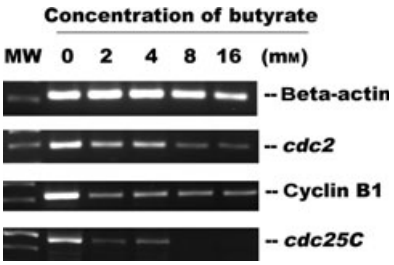

Fig. 5. Effect of butyrate on the expression of cell cycle-related genes (*cdc2*, *cdc25C* and cyclinB1) in gingival fibroblasts (GFs). GFs were exposed to different concentrations of butyrate for 24 h. Total RNA was isolated and used for RT-PCR analysis of cellular gene expression. One representative PCR image is shown. MW, molecular weight.

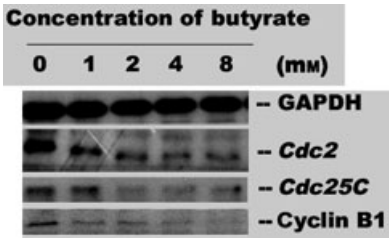

Fig. 6. Effect of butyrate on the expression of cell cycle-related proteins (Cdc2, Cdc25C and cyclinB1) in gingival fibroblasts (GFs). GFs were exposed to butyrate (1–8 mM) for 24 h. Equal amount of proteins from cell lysates were used for western blotting. One representative western blot is shown.

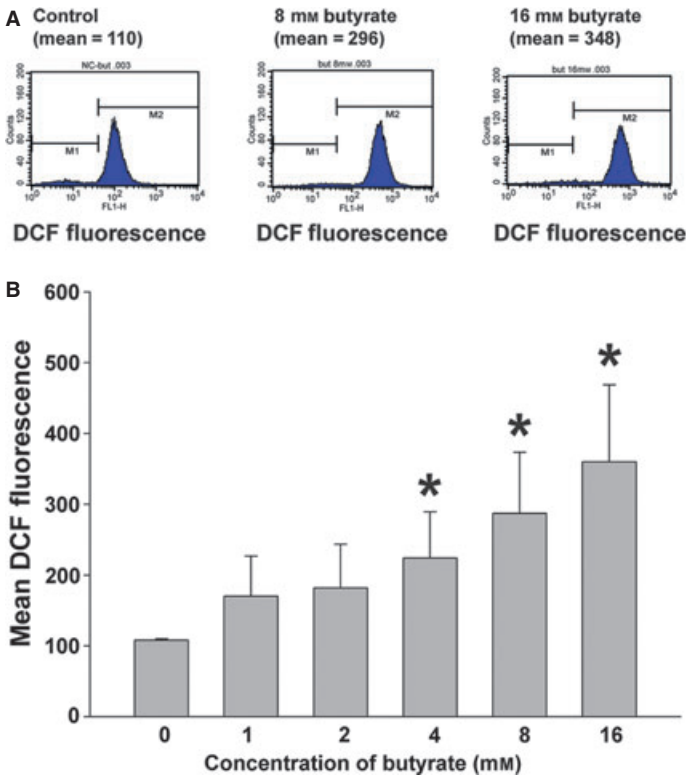

Fig. 7. Effect of butyrate (1-16 mM) on the cellular reactive oxygen species (ROS) level of gingival fibroblasts (GFs). (A) One representative histogram of 2',7'-dichlorofluorescein (DCF) fluorescence in control GFs and in GFs exposed to 8 and 16 mM butyrate. A shift of DCF fluorescence (FL1-H) to the right was noted, indicating an increase of DCF fluorescence. (B) Quantitative DCF fluorescence of GFs after exposure to various concentrations of butyrate ($n = 8$). The results are expressed as mean ± standard error of the mean DCF fluorescence. *Statistically significant difference compared with the untreated control ($p < 0.05$).

previous studies on GF growth. Factors such as culture medium (Dulbecco's modified Eagle's medium, alpha-minimum essential medium, or others), serum source (fetal calf serum or fetal bovine serum and their source), age of GF donors, passage, methods for primary culture (tissue explant technique or collagenase incubation) or population (Mongolian, etc.) may affect the proliferation of GFs (24–27). In this study, inhibition of GF growth by butyrate was not mainly caused by the induction of cell death. Whereas the numbers of viable GFs decreased following incubation for 24 h with butyrate, there was no marked increase in the numbers of floating cells and only a small population of GFs became apoptotic or necrotic after exposure to 16 mM butyrate, as revealed by the PI–annexin V dual-staining flow cytometric analysis. Accordingly, butyrate (< 5 mM) did not induce apoptosis of GFs cultured from healthy tissue, whereas it did elicit apoptosis of GFs cultured from inflamed tissue (13). Butyrate has also been shown to induce the apoptosis and autophagic cell death of gingival epithelial cells (28). These results reveal the differential response of cells to butyrate. The difference in the susceptibility of GFs obtained from healthy and inflamed gingiva to the toxic effect of butyrate may further lead to progression of periodontal destruction. Interestingly, butyrate induced arrest of GFs at G0/G1 and G2/M phases of the cell cycle, during exponential growth (Fig. 4) or in near-confluent culture (Fig. 3). The induction of cell cycle arrest by butyrate and other SCFAs produced by A. *actinomycetemcomitans* may inhibit cell proliferation and thereby be involved in the processes of gingival inflammation, wound healing and fibrosis (23,29).

Cell cycle control is critically regulated by a number of proteins, such as Cdc2, Cdc25C and cyclins. The G2/M transition is tightly regulated by Cdc2, Cdc25C and cyclinB1 (14,15,28). During mitosis, Cdc2 and cyclinB1 form a complex and then become dephosphorylated at T14 and Y15 by Cdc25C phosphatase for the full activation of kinase activity and cell cycle progression (30–32). However, little is known regarding the effect of butyrate on the cell cycle-related genes of oral mucosa cells. Interestingly, we found that butyrate down-regulates the expression of *cdc2*, *cdc25C* and cyclinB1 genes at the transcriptional level. Moreover, the levels of Cdc2, Cdc25C and cyclinB1 in GFs were also decreased after exposure

to butyrate. Butyrate, an inhibitor of histone deacetylation, was shown to inhibit the growth of liver cancer cells via down-regulation of Cdc2 and cyclin E (33) and cyclin B1 in oral cancer cells (8). In this study, we found that butyrate may suppress the expression of *cdc25C* at both transcriptional and translational levels. This may explain why exposure of GFs to butyrate may lead to cell cycle arrest.

The above inhibitory effect of butyrate on GFs may be associated with ROS production. The cytotoxicity and genotoxicity of a number of chemicals, such as cadmium and resin monomers, has been linked to oxidative stress (12,18,34). Elevation of ROS may deplete cellular glutathione, damage DNA, activate check-point kinases and regulate the cell cycle- and apoptosis-related genes (29,35). The levels of ROS are elevated in the inflammatory and early wound-healing processes as a defence against pathogens and to regulate angiogenesis, whereas lower levels of ROS may stimulate cell proliferation to promote wound closure (29). Clinical studies have found elevation of oxidant status and lipid peroxidation in the serum and the gingival crevicular fluid of patients with chronic periodontitis and evident periodontal bony destruction (12,36,37). Intriguingly, in this study, the ROS levels in GFs were elevated after exposure to butyrate. Stimulation of ROS production may involve the toxicity of butyrate and the progression of periodontal disease. Furthermore, the accumulation of butyrate in periodontal biofilms may stimulate inflammatory mediators or deregulate the host defense, contributing to periodontal destruction.

In conclusion, the results of the present study indicate that butyrate generated by periodontal pathogenic microorganisms exhibits a cytostatic effect via the suppression of cell cycle-related genes, such as *cdc2*, *cdc25C* and cyclinB1, leading to cell cycle arrest but not to apoptosis of GFs. This event may impair the repair and regeneration of periodontal tissue. The toxic effect of butyrate is associated with ROS production and contributes to the pathogenesis of periodontitis.

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