Original

Effects of sodium bicarbonate on butyric acid-induced epithelial cell damage *in vitro*

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Abstract: Butyric acid is detected in periodontal pockets and is thought to be involved in the initiation and progression of periodontal disease. We examined the effects of sodium bicarbonate on the butyric acid-induced epithelial cell damage. The human gingival carcinoma cell line Ca9-22 was cultured in medium that contained butyric acid with or without sodium bicarbonate. The viability of cells treated with sodium bicarbonate was significantly higher than that of cells treated with butyric acid alone. The effects of butyric acid on ICAM-1 expression were significantly improved by sodium bicarbonate. Within the limitations of this *in vitro* study, sodium bicarbonate was indicated to be a useful therapeutic agent to reduce the butyric acid-induced periodontal tissue damage. (J. Oral Sci. 50, 413-417, 2008)

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Introduction

Periodontitis is an inflammatory disorder that results in the destruction of the structures supporting the teeth. It is the most important cause of tooth loss among adults and is initiated by the accumulation of predominantly anaerobic Gram-negative bacteria in subgingival sites. Gram-negative anaerobes, such as *Porphyromonas gingivalis* and *Prevotella* and *Fusobacterium* spp. have been identified as important pathogens of periodontitis (1-3). Several virulence factors are associated with the pathogenicity of these bacteria, including lipopolysaccharide, fimbriae, hemagglutinin, hemolysin, and proteases (4-8). In addition, short-chain fatty acids such as butyric acid have been detected in periodontal pockets and are thought to be involved in the initiation and progression of periodontal disease (9). High concentrations of butyric acid have been found in the dental plaque of periodontitis patients (10-14). Takigawa et al. demonstrated that butyric acid significantly decreased viability of epithelial cells in a dose-dependent manner and induced ICAM-1 expression (15). Therefore, we postulate that the inhibition of the action of butyric acid on the epithelium reduces the pathogenicity of Gram-negative anaerobes.

In this study, we examined the effects of sodium bicarbonate on the influence of butyric acid on epithelial cells.

Materials and Methods

Cell culture

The human gingival carcinoma cell line Ca9-22 was obtained from the Health Science Research Resources Bank (Osaka, Japan) and cultured in minimum essential medium (Asahi Technoglass, Tokyo, Japan) that contained 10% fetal bovine serum (Asahi Technoglass). The medium was supplemented with 1% penicillin/streptomycin (Sigma, St. Louis, MO, USA). The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2. Cell suspensions were counted using a hemocytometer and seeded at 1×10^5 cells/well in six-well plates (Iwaki, Tokyo, Japan). After an initial 96-h incubation, the medium was replaced with fresh medium that contained 3 mM butyric acid alone or in combination with sodium bicarbonate ($NaHCO_3$, All Japan Drug, Tokyo, Japan) or fresh medium alone (control) and then incubated at 37°C in a humidified atmosphere of 5% CO_2. Butyric acid ($\geq$ 99%) was purchased from Sigma. Butyric acid has density 0.96 g/cm^3 and molecular mass 88.1; thus pure butyric acid is 10.9 M. The pH of medium containing 3 mM butyric acid was 7.3. No significant pH change was observed in this experimental condition.

Cell viability

The viable cells were counted after a final culture for 6 or 24 h. Viable cells were determined by Trypan blue exclusion assay. The data are expressed as the mean ± standard deviation of three independent experiments. Each experiment was performed in triplicate.

Scanning electron microscopy

The effects of butyric acid and sodium bicarbonate on cell morphology were evaluated using scanning electron

Fig. 1 Effects of butyric acid on the cell viability. Ca9-22 cells (1.0×10^5 cells/well) were cultured in 2-ml aliquots of medium containing 3 mM butyric acid with or without sodium bicarbonate for 6 or 24 h at 37°C in a humidified atmosphere of 5% CO_2.
* $P < 0.05$ vs control, + $P < 0.05$ vs 3 mM butyric acid

microscopy. After a 48 h initial incubation and a 24 h final incubation with butyric acid with or without sodium bicarbonate, the plates were dehydrated in an ascending ethanol series, processed in a Critical Point Dryer (HCP-2; Hitachi, Tokyo, Japan) and gold-coated using an Ion Coater (JFC-1100; JEOL, Tokyo, Japan). The processed samples were examined using scanning electron microscopy (TM-1000; Hitachi).

Real-time PCR analysis

After initial 96 h incubation and final 6 to 24 h incubation, the cells were harvested using 0.25% trypsin/EDTA and resuspended in phosphate-buffered saline (PBS). Total RNA was extracted from the cells using the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA). The cDNA was synthesized using the Ready-To-Go T-Primed First-Strand Kit (Amersham Biosciences, Piscataway, NJ, USA). Primer and probe sets for ICAM-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Applied Biosystems (Tokyo, Japan). Real-time PCR was performed on an ABI PRISM 7700 Sequence Detector (Applied Biosystems) using the following cycling parameters: 50°C for 2 min; 95°C for 10 min; 40 cycles of denaturation at 95°C for 15 s and primer extension at 60°C for 1 min. The gene expression levels were first normalized to that of GAPDH in the same sample, and the relative differences between the control and treatment groups were calculated and expressed as relative increases, setting the control at 100%.

Flow cytometry

After initial 96 h incubation and final 24 h incubation, the cells were harvested using 0.25% trypsin/EDTA and resuspended in PBS (pH 7.4). The cells were incubated with monoclonal anti-human ICAM-1 antibody (CD54-FITC; Immunotech, Marseille, France; at 20 μl antibody/10^6 cells). The suspension was incubated with gentle shaking for 30 min at 4°C in the dark. After each step, the cells were washed and resuspended in PBS. Flow cytometry was performed using EXPO32 (Beckman Coulter, Tokyo, Japan).

Statistical analysis

The results were evaluated using Student's *t*-test with Bonferroni correction. $P < 0.05$ was considered to be statistically significant.

Results

Effects of butyric acid and sodium bicarbonate on cell viability
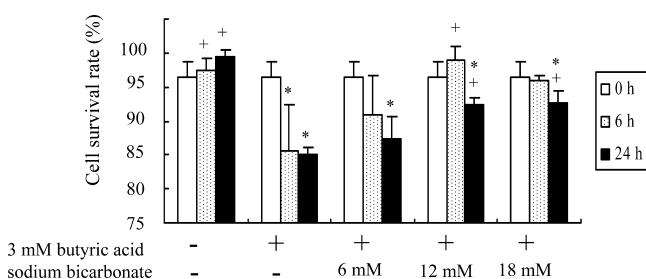
After culturing cells for 24 h in medium containing

butyric acid with or without sodium bicarbonate, the viability of the cells exposed to butyric acid alone was significantly lower than that of cells treated with butyric acid plus sodium bicarbonate (Fig. 1). The maximum effect was obtained with 12 mM sodium bicarbonate. Treatment with 12 mM sodium bicarbonate alone had no effect on cell viability. The pH of medium containing 3 mM butyric acid alone, 3 mM butyric acid with 12 mM sodium bicarbonate and 12 mM sodium bicarbonate alone were 7.3, 7.3 and 7.6, respectively.

Epithelial cell morphology

Control cells appeared as flat cells with thick cytoplasmic projections that adhered to the culture dish. Morphological changes with cell rounding were clearly observed for cells grown in the presence of butyric acid (Fig. 2). In contrast, thin cytoplasmic projections were observed for cells grown in the presence of butyric acid with sodium bicarbonate.

Effects of butyric acid and sodium bicarbonate on ICAM-1 expression

The effect of sodium bicarbonate on butyric acid-induced ICAM-1 expression was investigated using real-time PCR to monitor the levels of mRNA expression. In the presence of butyric acid, ICAM-1 expression was increased significantly at 6 h post-stimulation. In the presence of 48 mM sodium bicarbonate, ICAM-1 induction was significantly inhibited. However, no significant difference was observed at 24 h post-stimulation (Fig. 3). The pH of medium containing 3 mM butyric acid with 24 mM sodium bicarbonate and 48 mM sodium bicarbonate were 7.4 and 7.5. Similar induction was observed in the presence of butyric acid with and without sodium bicarbonate using flow cytometric analysis at 24 h post-stimulation (Fig. 4).

Discussion

Epithelial cells form a tight barrier that prevents mucosal penetration by bacterial pathogens. Butyric acid disrupts human cell adhesion and contributes to tissue damage in periodontal disease caused by Gram-negative anaerobes (9,15). This suggests that butyric acid in periodontal pockets disrupts the tight attachment of epithelial cells, resulting in bacterial penetration and periodontal tissue destruction. The viability of cells treated in combination with sodium bicarbonate was improved significantly compared to cells treated with butyric acid alone; sodium bicarbonate alone had no effect on ICAM-1 expression. ICAM-1 expression in the epithelium is thought to play an important role in leukocyte transendothelial migration to areas of inflammation in gingival tissue (16-18). Our results indicated that sodium bicarbonate significantly
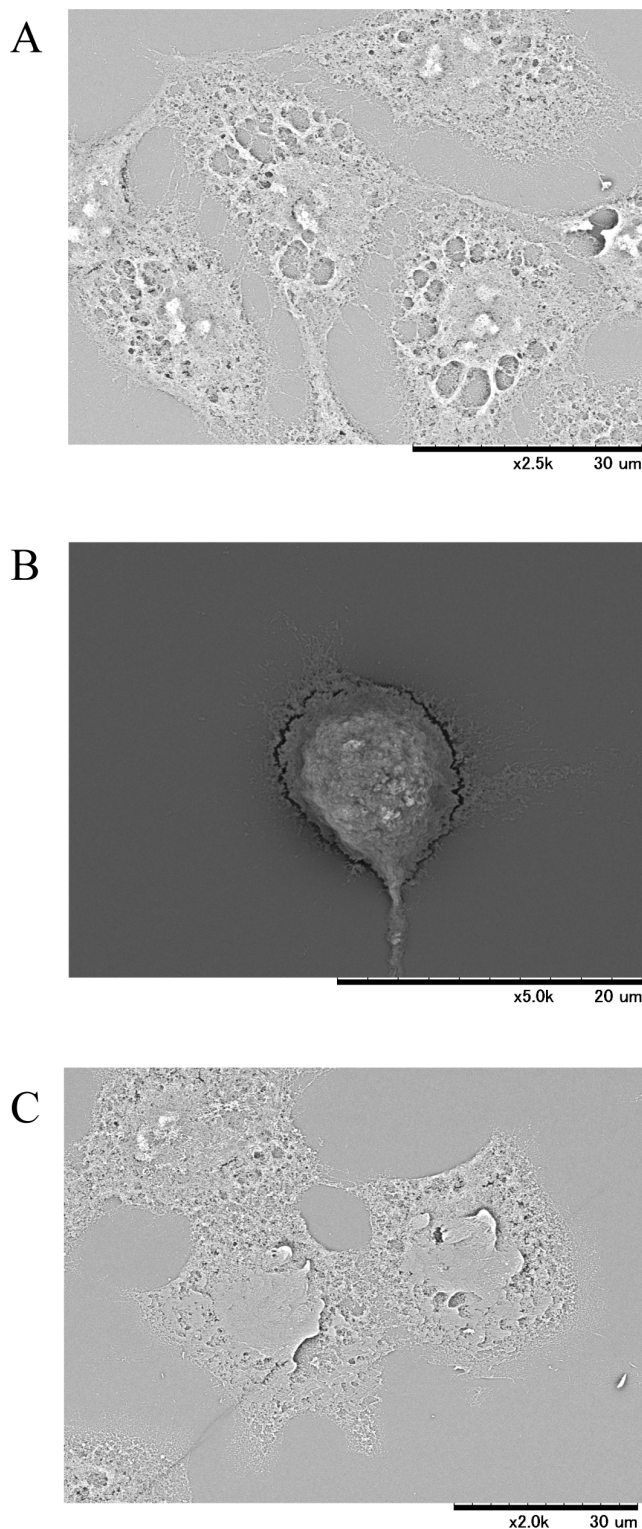
Fig. 2 Epithelial cell morphology. Cells were cultured in medium that contained 3 mM butyric acid and 12 mM sodium bicarbonate for 24 h. (A) Control; (B) 3 mM butyric acid; (C) 3 mM butyric acid and 12 mM sodium bicarbonate. Samples were examined using scanning electron microscopy at ×1,000 magnification.
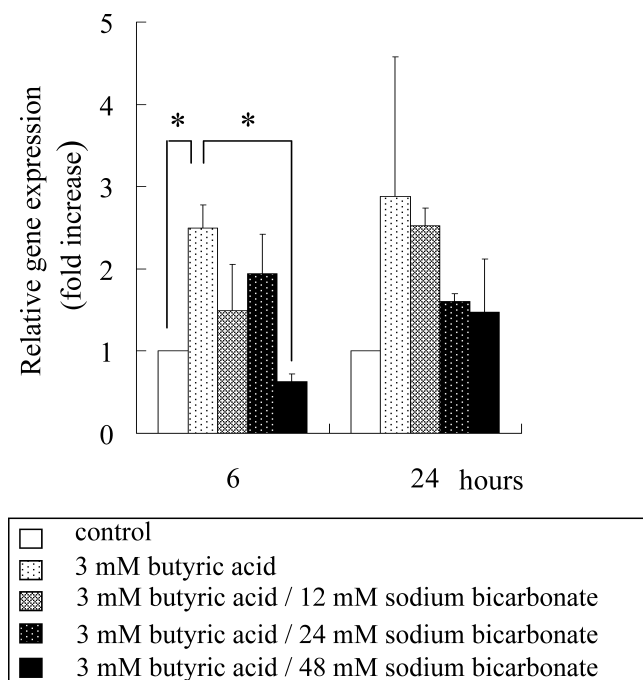
Fig. 3 Effects of butyric acid on ICAM-1 mRNA expression. Ca9-22 cells were incubated with medium that contained 3 mM butyric acid with or without 12 mM to 48 mM sodium bicarbonate for 6 h. Real-time PCR was used to monitor the levels of ICAM-1, which were normalized to GAPDH levels. The relative differences between the control and treatment groups are expressed as relative increases, with the control set at 100%. Relative gene expression is expressed as the mean ± SD of three independent experiments; * $P < 0.05$.
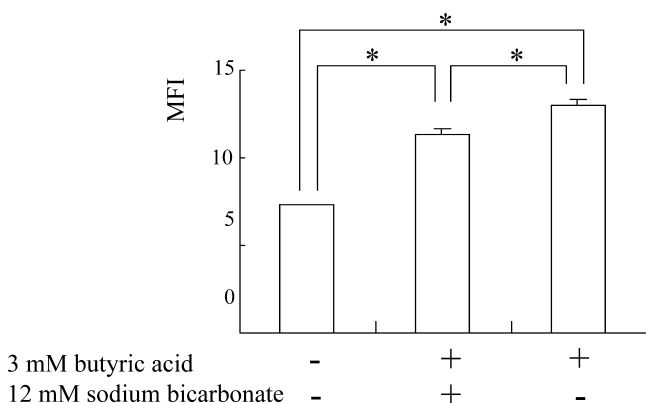

Fig. 4 Effects of butyric acid on the expression of ICAM-1. Cells were incubated with medium that contained 3 mM butyric acid with or without 12 mM sodium bicarbonate for 24 h. Flow cytometry was used to monitor the levels of ICAM-1. The mean fluorescence intensity (MFI) is expressed as the mean ± SD of three independent experiments; * $P < 0.05$.

inhibited butyric acid-induced inflammatory response.

Sodium bicarbonate was widely used as a dentifrice before the introduction of modern toothpastes. The merit of sodium bicarbonate products seems to lie in its safety, low cost, low abrasivity, water solubility, buffering ability, compatibility with fluoride, and antibacterial properties in high concentrations. Sodium bicarbonate-containing dentifrice exhibits greater bactericidal activity against *Streptococcus mutans* than other dentifrice (19,20). Christersson et al. reported that sodium bicarbonate packed subgingivally enhances periodontal healing when placed in periodontal pockets in conjunction with scaling and root planning procedures (21). A 12 month post- treatment, 81% of the sites that were initially 7 mm or deeper showed a 2 mm or more gain in clinical attachment after placing adjunctive sodium bicarbonate. Our results suggest that the neutralization of butyric acid accounts, at least in part, for the clinical benefit of sodium bicarbonate application.

In summary, within the limitations of this *in vitro* study, sodium bicarbonate appears to be a useful therapeutic agent to reduce the butyric acid-induced periodontal tissue damage.

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