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The effect of butyric acid on adhesion molecule expression by human gingival epithelial cells

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Background and Objective: Short-chain fatty acids, such as butyric acid, are detected in periodontal pockets and are thought to be involved in the initiation and progression of periodontal disease. In the present study, we examined the effects of butyric acid on adhesion molecule expression by human gingival epithelial cells.

Material and methods: The human gingival carcinoma cell line, Ca9-22, was cultured in media that contained different concentrations of butyric acid.

Results: Cell numbers were significantly decreased in a dose-dependent manner by butyric acid at concentrations of ≥ 0.2 mM. The expression of intercellular adhesion molecule-1 mRNA was significantly increased 6 h after stimulation. By contrast, the expression levels of integrins α6 and β4 were decreased. Similar results were obtained by flow cytometry.

Conclusion: The results of the present study indicate that butyric acid alters the expression of adhesion molecules by Ca9-22 cells. The elucidation of the mechanism of action of butyric acid on the periodontium may help to clarify several aspects of the onset and progression of periodontal disease.

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Adult periodontitis is a chronic infectious disease induced by gram-negative bacteria. Gram-negative anaerobes, such as *Porphyromonas gingivalis, Prevotella* and *Fusobacterium* spp., have been identified as important periodontal pathogens (1–4). Several virulence factors, including lipopolysaccharide, fimbriae, hemagglutinin, hemolysin and proteases, have been associated with the pathogenicities of these bacteria (5–8). 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). Butyric acid concentrations in the gingival crevicular fluid have been shown to correlate with gingival inflammation and periodontal pocket depth. Furthermore, millimolar levels of butyric acid have been found in the dental plaque of patients with periodontitis (10–14). Previous studies have shown that butyric acid inhibits the growth of gingival fibroblasts and epithelial cells and induces apoptosis in T and B cells (15–17). A previous study has demonstrated that butyric acid significantly retards epithelial cell growth. In addition, accumulation of a dense population of intermediate fibrils, filaments and cytoplasmic vacuolization have been observed in epithelial cells after treatment with butyric acid. However, the precise mechanisms of action of butyric acid on epithelial cells have not been elucidated.

In the present study, we examined the effects of butyric acid on the expression of adhesion molecules by human gingival epithelial cells.

Material 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. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2.

Cell proliferation

Cell suspensions were counted by use of a hemocytometer and were seeded at 1×10^5 cells/well in six-well plates (Iwaki, Tokyo, Japan). Cells were cultured in 2-mL volumes of different concentrations of 0.02–3.0 mM butyric acid (Sigma, St Louis, MO, USA) for 7 d at 37°C in a humidified environment of 5% CO_2. Cell numbers were estimated every 24 h. The data are expressed as the means ± standard deviation from three independent experiments. Each experiment was performed in triplicate.

Scanning electron microscopy

The effects of butyric acid on cell morphology were evaluated by scanning electron microscopy. Cells were seeded at 1.0×10^5 cells/well in six-well plates. After 48 h of incubation, the medium was replaced with either fresh medium that contained 3.0 mM butyric acid or fresh medium alone (control) for 24 h. After treatment, 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 by scanning electron microscopy (TM-1000; Hitachi).

Real-time polymerase chain reaction analysis

Cells were seeded at 1.0×10^5 cells/well in six-well plates. After 96 h of incubation, the medium was replaced with either fresh medium that contained 3.0 mM butyric acid or fresh medium alone (control) for 6 h at 37°C in a humidified atmosphere of 5% CO_2. After treatment, the cells were harvested using 0.25% trypsin/EDTA and resuspended in phosphate-buffered saline.

Total RNA was extracted from the cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). cDNA was synthesized with the Ready-To-Go T-Primed First-Strand Kit (Amersham Biosciences, Piscataway, NJ, USA). Primer and probe sets for intercellular adhesion molecule-1, integrin α6 and integrin β4 were obtained from Applied Biosystems (Tokyo, Japan). Real-time polymerase chain reaction analysis was performed on an ABI PRISM 7000 Sequence Detector (Applied Biosystems), with the following cycling parameters: 50°C for 2 min, 95°C for 10 min, followed by 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 glyceraldehyde-3-phosphate dehydrogenase 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%. Data are shown as the means ± standard deviation of three independent experiments.

Flow cytometry

Cells were seeded at 1.0×10^5 cells/well in six-well plates, as described in the previous section. After 96 h of incubation, the medium was replaced with either fresh medium that contained 3.0 mM butyric acid or fresh medium alone (control) for 24 h at 37°C in a humidified atmosphere of 5% CO_2. After treatment, the cells were harvested using 0.25% trypsin/EDTA and then resuspended in phosphate-buffered saline (pH 7.4). The cells were incubated with one of the following antibodies (at 20 μL of antibody per 10^6 cells): monoclonal anti-human intercellular adhesion molecule-1 (CD54-FITC; Immunotech, Marseille, France); monoclonal anti-human integrin α6 (CD104-PE; BD Biosciences, Tokyo, Japan); and monoclonal anti-human integrin β4 (CD49f-FITC; BD Biosciences). The suspension was incubated with gentle shaking for 30 min at 4°C in the dark. After each step, the cells were washed and suspended in phosphate-buffered saline. Flow cytometry was performed with EXPO32 (Beckman Coulter, Tokyo, Japan). Data shown are representative of the results of three independent experiments.

Statistical analysis

The results were evaluated using the Mann–Whitney *U*-test. A p-value of < 0.05 was considered to be statistically significant.

Results

Effects of butyric acid on Ca9-22 cell proliferation

To examine the effect of butyric acid on Ca9-22 proliferation, the cells were cultured in different concentrations of butyric acid. After culture in butyric acid the cell numbers were found to be inhibited in a dose-dependent manner (Fig. 1). In particular, cell growth was impaired 4 d after incubation in 0.2 and 0.4 mM butyric acid. In the presence of butyric acid at 1.0 and 3.0 mM, cell viabilities were 89.6 and 49.0% at 4 d, respectively. By contrast, a slight increase in Ca9-22 number was observed for cells grown in the presence of 0.02 mM butyric acid.

Epithelial cell in morphology

Control cells appeared as flat cells that adhered to the culture dish. Morphological changes with cell rounding were clearly observed for cells grown in the presence of 3.0 mM butyric acid (Fig. 2). These changes in cell morphology were observed for all the 3.0 mM butyric acid-exposed cells at 24 h.

Effects of butyric acid on intercellular adhesion molecule-1, integrin α6 and integrin β4 expression

Experiments were carried out to investigate the effect of butyric acid (3.0 mM) on adhesion molecule expression. Real-time polymerase chain reaction was used to monitor the

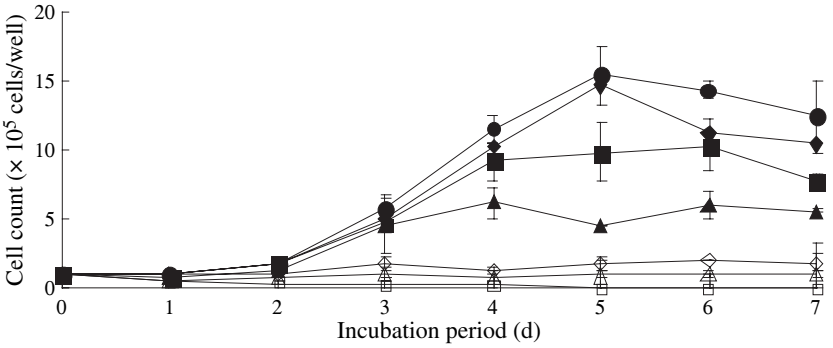

Fig. 1. Effects of butyric acid on cell proliferation. Ca9-22 cells (1.0×10^5 cells/well) were cultured in 2-mL aliquots of medium, containing different concentrations of butyric acid, for 7 d at 37°C in a humidified atmosphere of 5% CO_2. Cell numbers were assessed every 24 h. Butyric acid concentration: control (♦), 0.02 mM (•), 0.2 mM (■), 0.4 mM (▲), 0.8 mM (◇), 1.0 mM (△), 3.0 mM (□).

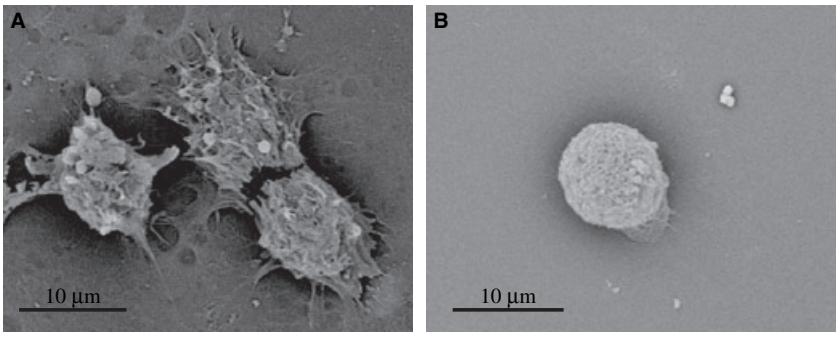

Fig. 2. Epithelial cell morphology. Ca9-22 cells were cultured in medium, with (B) or without (A) 3.0 mM butyric acid, for 24 h. Samples were examined by scanning electron microscopy at ×1000 magnification. Cell viabilities with or without butyric acid were 84.8 and 81.5%, respectively.

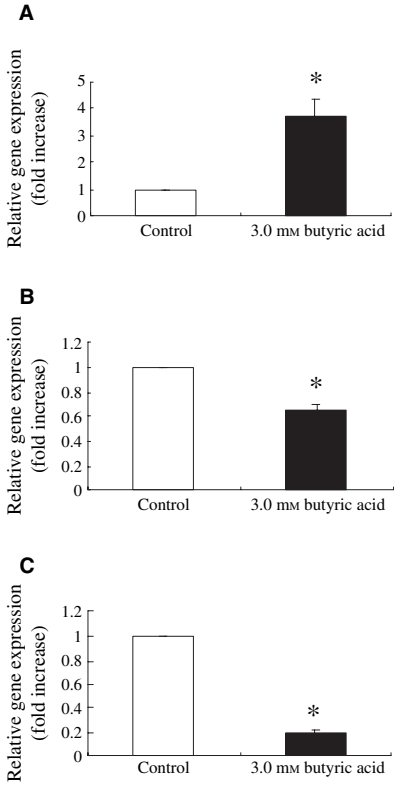

Fig. 3. Effects of butyric acid on intercellular adhesion molecule-1, integrin α6 and integrin β4 mRNA expression. Ca9-22 cells were incubated for 6 h in medium that contained butyric acid. Real-time polymerase chain reaction was used to monitor the levels of intercellular adhesion molecule-1 (A), integrin α6 (B) and integrin β4 (C) mRNA, which were normalized to that of glyceraldehyde-3-phosphate dehydrogenase; the relative differences between the control and treatment groups are expressed as relative increases, with the control set at 100%. Control (□), 3.0 mM butyric acid (■). *Significantly different between with and without butyric acid ($p < 0.05$).

levels of Ca9-22 transcripts. In the presence of 3.0 mM butyric acid, intercellular adhesion molecule-1 expression was significantly increased 6 h after stimulation. By contrast, the expression levels of integrin α6 and integrin β4 were decreased at this time-point (Fig. 3). Similar results were observed in flow cytometric analyses (Fig. 4). After 24 h of incubation with 3.0 mM butyric acid, cell viability was 81.5%.

Discussion

It has previously been reported that butyrate inhibits epithelial cell growth, spreading and proliferation at concentrations commonly found in plaque and gingival crevicular fluid during inflammation (3). The present study shows that butyric acid at concentrations of ≥ 0.2 mM inhibits cell proliferation in a dose-dependent manner. By contrast, proliferation showed a slight increase for cells cultured in 0.02 mM butyric acid. A dual effect was observed for intestinal cells. Indeed, at low concentrations (0.05 and 0.1 mM), butyrate stimulated cell proliferation weakly. At 0.5 mM butyric acid, no modulation of proliferation was observed, whereas butyric acid concentrations of ≥ 1 mM inhibited cell proliferation in a dose-dependent manner (18, 19). Interestingly, similar results have been obtained for oral epithelial cells. Ca9-22 cells, although derived from gingival carcinoma, were chosen because they are a well-established model for periodontal research (20–22).

In the present study, we further investigated the ability of butyric acid to modulate the expression of adhesion molecules in epithelial cells. Intercellular adhesion molecule-1 plays an important role in the transendothelial migration of leukocytes to areas of inflammation in gingival tissue. In the gingival epithelium, intercellular adhesion molecule-1 is expressed in the junctional epithelium and sulcular epithelium, forming a concentration gradient, with the highest level on the epithelium that faces the tooth surface. This gradient is thought to play an important role in directing the migra-

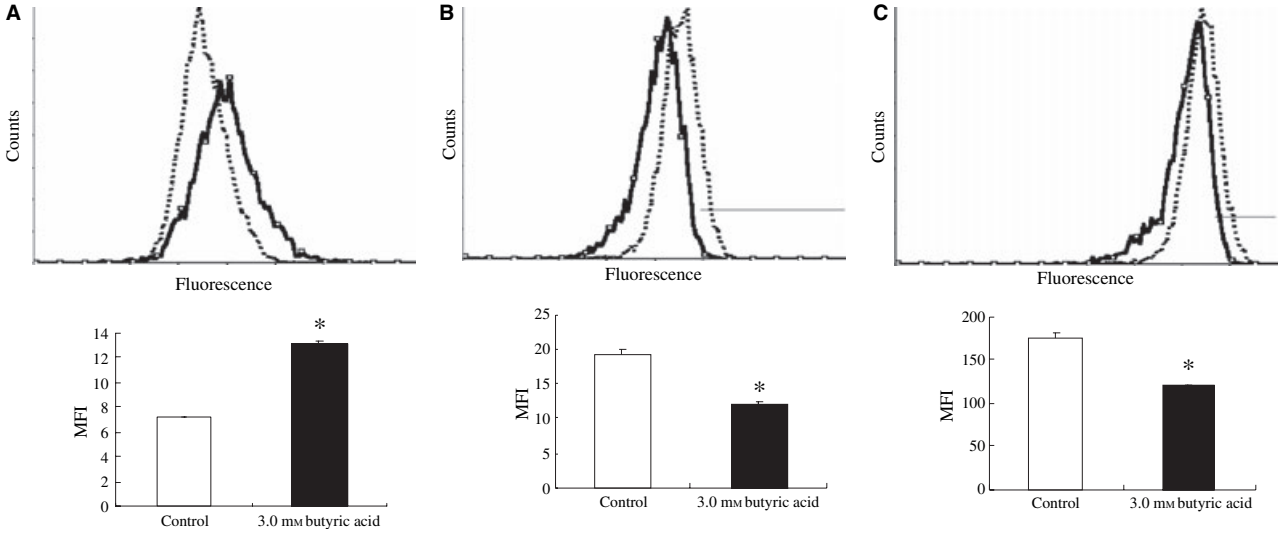

Fig. 4. Effects of butyric acid on the levels of intercellular adhesion molecule-1, integrin α6 and integrin β4. Cells were incubated for 24 h in medium that contained 3 mM butyric acid. Flow cytometry was used to monitor the levels of intercellular adhesion molecule-1 (A), integrin α6 (B) and integrin β4 (C). The solid line and the dotted line indicate 3.0 mM butyric acid and the control, respectively. Mean fluorescence intensity (MFI) is expressed as mean ± SD of three independent experiments. *Significantly different between with and without butyric acid ($p < 0.05$).

tion of inflammatory cells towards the sulcular space. These observations indicate that butyric acid augments inflammatory cell migration. (23–26).

Epithelial cells form a tight barrier that prevents mucosal penetration by bacterial pathogens. The attachment of the gingiva to the tooth surface is mediated by the junctional epithelium, which is a specialized tissue that forms a barrier between periodontal connective tissues and the oral cavity. Cellular integrins mediate epithelial barrier formation, as well as cell activation, proliferation and differentiation (27, 28). The integrins represent a large family of transmembrane receptor glycoproteins, which are structurally diversified according to function and cell-type specific expression and which can interact with a complex spectrum of ligands. Integrins are heterodimers that are formed by noncovalent association of the glycoprotein α and β subunits (29–32). The β4 subunit is located mainly at the cell membrane, facing the inner and outer basal laminae in junctional epithelium, and located at the basal surfaces of basal cells in other parts of the gingival epithelium. The α6 subunit has a wider distribution than the β4 subunit, being expressed both at cell–matrix contacts and at the cell–cell contacts of the junctional epithelium. Therefore, the α6 and β4 subunits at dento-epithelial junctions are essential for the cell–matrix or cell–cell interactions that maintain the tissue architecture (33, 34). In the present study, butyric acid treatment significantly reduced the expression levels of the α6 and β4 subunits. These results suggest that butyric acid in periodontal pockets may disable the tight attachment among epithelial cells, resulting in bacterial penetration and periodontal tissue destruction.

The most commonly reported mechanism by which butyric acid modulates gene expression involves an alteration of chromatin structure subsequent to increased histone acetylation. Butyric acid potently inhibits histone deacetylase activity, which leads to histone hyperacetylation. It is generally assumed that histone hyperacetylation results in relaxation of the chromatin structure, thereby making DNA accessible to a variety of transcription factors. A variety of biological phenomena induced by butyric acid may be ascribed to histone hyperacetylation. However, the role of histone acetylation in butyrate-related biological phenomena needs to be clarified (35, 36).

In conclusion, the results of the present study show that butyric acid alters the expression of adhesion molecules by Ca9-22 cells. Elucidation of the mechanism of action of butyric acid on the periodontium may help to clarify several aspects of the onset and progression of periodontal disease and may suggest new therapeutic approaches.

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