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Normal human gingival fibroblasts undergo cytostasis and apoptosis after long-term exposure to butyric acid

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ABSTRACT

The causes of periodontal disease are complex. Butyric acid, a metabolite of periodontopathic bacteria such as *Porphyromonas gingivalis*, acts as a histone deacetylase inhibitor that has a direct effect on mRNA expression. Butyric acid produced by *Clostridium butyricum* in the intestinal tract induces differentiation of regulatory T cells, thereby suppressing inflammation in the gut. Mice lacking *Clostridium butyricum* in the intestinal tract suffer from colitis. By contrast, butyric acid in the oral cavity worsens periodontal disease. Periodontal disease is a chronic condition in which periodontal tissue is exposed to virulence factors (such as butyric acid); however, no study has examined the effects of long-term exposure to butyric acid. The present study demonstrated that long-term exposure of human gingival fibroblasts (HGFs) to butyric acid induced cytostasis and apoptosis via the intrinsic and extrinsic pathways. Butyric acid inhibited the division of HGFs by altering expression of mRNAs encoding cyclins. Butyric acid induced apoptosis in HGFs via the intrinsic pathway, followed by activation of caspase 9; there was no DNA damage or p53 activation. Butyric acid also upregulated expression of TNF-α mRNA and protein by HGFs. Furthermore TNF-α induced apoptosis by activating caspase 8 (the extrinsic pathway) and by inducing production of pro-inflammatory cytokines. Taken together, the results show that butyric acid induced cytostasis and apoptosis in HGFs, accompanied by production of pro-inflammatory cytokines. It thus acts as a death ligand and plays a critical role as a prophlogistic substance.

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1. Introduction

Periodontal disease is a chronic disease that causes inflammation of periodontal tissue and subsequent tooth loss [1]. A majority of people all over the world have periodontal disease; indeed, the prevalence rate in those aged 65 years and older is more than 60% [2]. Periodontal disease is often painless during the early stages; therefore, it is mostly identified at an advanced stage and carries a high risk of tooth loss, along with other symptoms such as throbbing pain and tooth mobility [3]. Tooth loss results in a marked decline in QOL due to both functional and esthetic reasons [4]. Furthermore, many studies suggest that advanced periodontal disease correlates with the presence of other serious systemic diseases [5] such as diabetes mellitus [6], vascular disease [7], heart disease [8], and rheumatic disease [9]. Thus, the causes of periodontal disease must be identified to prevent/abrogate systemic inflammation.

Anaerobic gram-negative bacteria in subgingival plaques harbor many virulence factors that affect periodontal disease progression [10]. *Porphyromonas gingivalis* is one type of periodontopathic bacteria that produces short chain fatty acids, mainly butyric acid, as a metabolite. Butyric acid also acts as a histone deacetylase (HDAC) inhibitor that affects mRNA expression [11]. Butyric acid produced by intestinal bacteria induces expression of Foxp3 and subsequent differentiation of regulatory T cells, thereby suppressing inflammation [12]. By contrast, high concentrations of butyric acid in the oral cavity correlate with periodontal tissue destruction [13]. The amount of butyric acid in the gingival sulcus correlates with the stage of periodontal disease [14,15] and high concentrations of butyric acid induce apoptosis in inflamed human gingival fibroblasts (HGFs) [16]; however, few studies have examined the

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direct effects of butyric acid on periodontal tissue.

Gingival fibroblasts are a major constituent of periodontal tissue and as such are likely to be exposed to butyric acid at the gingival sulcus. Long-term exposure to butyric acid may contribute to periodontal tissue destruction over time; indeed, periodontal disease is a chronic condition. Here, we examined the effects of long-term exposure to butyric acid on normal HGFs.

2. Materials and methods

2.1. Cells

RAW264.7, a mouse macrophage-like cell line, and primary human gingival fibroblasts (HGFs) were purchased from the Riken cell bank (Tsukuba, Japan) and ScienCell Research Laboratories (California, America), respectively. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, Missouri, USA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL Streptomycin, and 0.25 μg/mL Amphotericin B. HGFs were used between passages three and ten.

2.2. Cell viability assay

Butyric acid (Sigma-Aldrich) was diluted in DMEM and adjusted to pH 7.2–7.4 with sodium hydroxide. RAW264.7 and HGFs were treated with different concentrations of butyric acid (0, 1.25, 2.5, 5, 10, and 15 mM) at 37 °C under a humidified condition of 5% CO_2 for 24, 48, or 72 h. Cell viability was then measured in an MTT assay using Cell Count Reagent SF (Nacalai Tesque, Kyoto, Japan), following manufacturer's protocol.

2.3. Measurement of cell division

HGFs were suspended in PBS at a density of 1×10^6 cells/mL and stained with 1 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Affymetrix, California, USA) for 5 min at room temperature. HGFs were then washed three times in cold DMEM containing 10% FBS. CFSE-labeled HGFs were then treated with different concentrations of butyric acid (0, 1, 5, and 15 mM). Fluorescence intensity of CFSE was measured on Days 0, 1, 2, and 3 using FACSCalibur flow cytometer (Becton-Dickinson, Mansfield, MA, USA) and Win-MDI software, version 2.9 (developed by Joe Trotter).

2.4. Real-time RT-PCR

HGFs were treated with butyric acid (0, 1, 5 or 15 mM) for 24, 48, or 72 h. Total RNA was then extracted using ISOGEN II (NIPPON GENE, Tokyo, Japan), followed by reverse-transcription using ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan). cDNA was amplified and quantified by real-time PCR using Applied Biosystems 7300 Real Time PCR system (Thermo Fisher Scientific) with Power SYBR Master Mix (Thermo Fisher Scientific) and 0.5 μM each of specific primers (Table 1). The thermal cycling conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 45 amplification cycles at 95 °C for 15 s and then 60 °C for 1 min.

2.5. Analysis of apoptosis

HGFs were exposed to butyric acid (0, 1, 5, and 15 mM) for 48 or 72 h and stained with AnnexinV-FITC (AnnexinV FLUOS Staining Kit) (Roche), propidium iodide (PI) (AnnexinV FLUOS Staining Kit), and Hoechst 33342 (Sigma-Aldrich) for 15 min at room temperature. Apoptosis was evaluated by FACSCalibur Flow Cytometer (Becton-Dickinson) and fluorescence microscopy BZ-X700 (KEYENCE, Osaka, Japan).

Table 1
Specific primers for real-time RT-PCR.

Target	Primer	Sequence
GAPDH	Forward	5′-AGCCACATCGCTCAGACAC-3′
	Reverse	5′-GCCCAATACGACCAAATCC-3′
IL-1β	Forward	5'-CTGTCCTGCGTGTTGAAAGA-3'
	Reverse	5′-TTGGGTAATTTTTGGGATCTACA-3′
IL-6	Forward	5′-GAAGCTCTATCTCGCCTCCA-3′
	Reverse	5′-AGCAGGCAACACCAGGAG-3′
TNF-α	Forward	5′-AGCCTCTTCTCCTTCCTGAT-3′
	Reverse	5′-GCCAGAGGGCTGATTAGAGA-3′
Cyclin A2	Forward	5′-GGTACTGAAGTCCGGGAACC-3′
	Reverse	5′-GAAGATCCTTAAGGGGTGCAA-3′
Cyclin B1	Forward	5′-ACATGGTGCACTTTCCTCCT-3′
	Reverse	5′-AGGTAATGTTGTAGAGTTGGTGTCC-3′
Cyclin D2	Forward	5′-GGACATCCAACCCTACATGC-3′
	Reverse	5′-CGCACTTCTGTTCCTCACAG-3′
Cyclin E2	Forward	5′-GCCATTGATTCATTAGAGTTCCA-3′
	Reverse	5′-CTGTCCCACTCCAAACCTG-3′
p53	Forward	5′-AGGCCTTGGAACTCAAGGAT-3′
	Reverse	5′-CCCTTTTTGGACTTCAGGTG-3′
Bak	Forward	5′-TGGTCACCTTACCTCTGCAAC-3′
	Reverse	5′-ATGTCGTCCCCGATGATG-3′

2.6. Caspase activity assay

Caspase 8, caspase 9, and caspase 3/7 activity in HGFs treated with or without 5 mM butyric acid was measured at 48 or 72 h using the Caspase-Glo8 Assay (Promega, Wisconsin, USA), Caspase-Glo9 Assay (Promega), or Caspase-Glo3/7 Assay (Promega), respectively, following manufacturer's protocols.

2.7. Detection of DNA double strand breaks (DSBs)

HGFs were exposed to butyric acid for 72 h. Positive control cells were stimulated with etoposide for 1 h before staining. DSBs were then detected by staining for γH2A using the OxiSelect™ DNA Double strand Break Staining Kit (CELL BIOLABS, California, USA), following manufacturer's protocols. After Hoechst 33342 staining, DSBs were observed under a fluorescence microscope.

2.8. ELISA

HGFs were treated (or not) with 15 mM butyric acid for 72 h and TNF-α in the culture supernatant measured using a Human TNF-α Quantikine ELISA kit (R&D systems, Minnesota, USA), following manufacturer's protocols.

2.9. TNF-α neutralization test

A monoclonal antibody specific for human TNF-α (clone 5N) was purchased from Hycult biotech (Netherlands). HGFs were exposed to 15 mM butyric acid for 48 or 72 h in the presence/absence of the anti-TNF-α antibody. Apoptosis, caspase 8 activity at 48 h, and mRNA expression at 72 h were then evaluated using the methods described above.

2.10. Statistical analysis

Data are expressed as the mean ± SE. All statistical analyses were performed using ANOVA with Tukey's test or using Student's t-test. $P < 0.05$ was considered statistically significant.

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