**Butyric acid induces apoptosis in both human monocytes and lymphocytes equivalently**

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**Abstract:** Short-chain fatty acids (SCFAs) are metabolites from anaerobic periodontopathic bacteria that induce apoptosis in immune cells such as lymphocytes, monocytes and macrophages. However, it remains unclear if SCFAs from pathogens induce apoptosis in monocytes/macrophages similarly with lymphocytes. This study investigated whether SCFAs-induced apoptosis is equal among the immunoregulatory cells. Cell apoptosis of the employed human cells was evaluated after treatment with culture supernatants from various periodontopathic bacteria or sodium butyrate. Apoptosis and viability were determined by detection of DNA fragmentation and using an MTS assay kit, respectively. *Porphyromonas gingivalis* and *Fusobacterium nucleatum* culture filtrates strongly induced apoptosis whereas *Prevotella nigrescens* and *Prevotella intermedia* culture filtrates failed to induce apoptosis in the THP-1 and U937 human monocyte and macrophage cell lines. Healthy gingival fibroblasts and oral epithelial cells were resistant to all the culture filtrates. Gas-liquid chromatography detected butyric acid in *P. gingivalis* (21.0-34.0 mM) and *F. nucleatum* (36.0 mM) in culture filtrates, whereas, only trace levels were seen in *P. nigrescens* and *P. intermedia*. These results suggest that butyric acid produced by periodontopathic bacteria severely damages immunoregulatory cells in a consistent manner and, likewise, could be involved in mediating periodontal chronic inflammation. (J Oral Sci 54, 7-14, 2012)

Keywords: apoptosis; butyric acid; leukocyte; periodontopathic bacteria.

**Introduction**

Periodontal diseases are infectious and cause periodontal tissue breakdown as a result of anaerobic bacteria specific interaction and host immune mechanisms (1-3). We previously demonstrated that short-chain fatty acids (SCFAs), an extracellular metabolites from periodontopathic bacteria, greatly inhibit proliferation and cytokine production of T and B cells (4). Among SCFAs, butyric acid particularly induces apoptosis in mouse and human T cells (5), B-cells (6), monocytes and macrophages (7), whereas, oral epithelial cells and healthy gingival fibroblasts (8) are resistant to butyric acid-induced apoptosis. Interestingly, inflamed gingival fibroblasts from adults with periodontitis are highly susceptible to butyric acid-induced apoptosis (9). Butyric acid concentration in subgingival plaque and in the periodontal pocket can range from 2.6-8.2 mM (10,11) and, in particular, its concentration in the periodontal pocket is correlated with periodontal disease severity (12). These findings are consistent with the pathogenic role of SCFAs, especially butyric acid, in periodontal diseases.

Contrasting leukocyte and non-leukocyte susceptibilities to butyric acid-induced apoptosis may be due to cell lineage difference. Monocytes, macrophages and lymphocytes develop separately from a common myeloid and lymphoid progenitor, respectively which may suggest that different types of immune cells have variable SCFAs-susceptibility. However, to date, no
studies have compared the susceptibility of butyric acid-induced apoptosis between macrophages/monocytes and lymphocytes.

Periodontopathogens also produce a variety of virulence factors such as proteases (13), lipopolysaccharide (LPS) (14), and fimbria (3,15), as well as SCFAs. Even if monocytes/macrophages have varying butyric acid-susceptibility to other immune cells, it is still unclear whether apoptosis is induced in monocytes/macrophages as well as lymphocytes by pathogens. In this regard, the effect of soluble factors produced by periodontopathic bacteria on apoptosis of monocytes/macrophages has not been studied in detail.

Here, the effects of periodontopathic bacteria culture filtrates were compared with regard to human monocyte/macrophage, lymphocyte, fibroblast and epithelial cell apoptosis. It was hypothesized that butyric acid present in the supernatant is a major factor accounting for apoptosis in immune cells.

**Materials and Methods**

**Human cell cultures**

Human Jurkat T lymphoma, U937 and THP-1 monocyte/macrophage, and Raji B lymphoma cell lines were cultured in complete medium consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µM 2-mercaptoethanol. Healthy human gingival fibroblast cell line N23 and the oral epithelial cell line Ca9-22 were cultured in complete medium consisting of Dulbecco’s modified Eagle’s medium supplemented with 10% FCS and antibiotics. All cell lines were cultured at 37°C in humidified air containing 5% CO₂.

**Preparation of culture filtrates**

*Porphyromonas gingivalis; ATCC 33277, W83, W50,* *Prevotella nigrescens* *ATCC 33563, Prevotella intermedia* *ATCC 25611,* *Fusobacterium nucleatum* *ATCC 23726,* and *Aggregatibacter actinomycetemcomitans* *Y4* were used throughout this study. *P. gingivalis* W83 and W50 were kindly provided by Dr. K. Okuda, Tokyo Dental College, Japan. All strains were independently grown in BHI-HMS medium consisting of brain heart infusion broth (BHI, Difco Laboratories, Detroit, MI, USA) supplemented with 5% FCS, 5 µg of hemin/ml and 0.5 µg of menadion/ml at 37°C in the anaerobic system model 1024 (Forma Scientific, Marietta, OH, USA) for 2 days. The cultures were centrifuged at 7,800 × g for 40 min at 4°C. The supernatant fluid was filtrated using a 0.22-µm-pore-size membrane filter (Millipore Corp., Bedford, MA, USA). Sterile BHI-HMS broth was used as control in all studies.

**Apoptosis induction**

Lymphocytes and monocytes (1 × 10⁶ cells/well) were cultured in 1 ml complete medium using 24-well tissue-culture plates (Falcon; Becton Dickinson Labware, Lincoln Park, NJ, USA). Fibroblasts and epithelial cells (5 × 10⁵ cells/well) were cultured in 2 ml of the complete medium using 6-well tissue-culture plates. Cells were incubated for 24 h either 10% (v/v) of bacterial culture filtrates or BHI-HMS broth with sodium butyrate (SB, 1.25-5.0 mM; Wako, Tokyo, Japan).

**DNA fragmentation assay and gel electrophoresis**

Cell harvesting and lysis of cells, diphenylamine (DPA) reaction, and gel electrophoresis were performed, as described previously (5). Briefly, cells were lysed in 400 µl hypotonic lysis buffer (0.2% Triton-X-100, 10 mM Tris, and 1 mM EDTA, pH 8.0) and centrifuged. Half of the supernatant, after cell lysis, was used in DPA reaction and the other half was subjected to gel electrophoresis. DPA reaction was performed to quantify the amount of uncut DNA in the pellets or fragmented DNA in the supernatant. Colorimetric reaction was quantified spectrophotometrically at 575 nm. Percent fragmentation was calculated as the ratio of DNA in the supernatants to the total DNA. Fragmented DNA was precipitated with isopropyl alcohol and, subsequently, resuspended in Tris-EDTA solution and gel electrophoresis.

**Cell proliferation and viability assay**

Cells (1 × 10⁴ cells/well) were treated in 0.1 ml complete medium containing either the culture filtrates or SB as described above in flat-bottomed 96-well plates. After 24 h, cell proliferation and viability were measured using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA).

**SCFAs measurement**

SCFAs present in the culture filtrates were analyzed using a gas-liquid chromatograph (GC-2014AF, Shimadzu, Tokyo, Japan) equipped with a hydrogen flame ionization detector. Measurements were repeated three times and the mean values were calculated.

**Statistical analysis**

Multiple-group comparisons were made by one-way analysis of variance, followed by post hoc intergroup comparisons using the Dunnett test. Where appropriate,
the Student’s t-test was used to assess the statistical significance of the differences between two groups.

Results
Effects of periodontopathic bacteria culture filtrates on T cell apoptosis
The pH range of *P. gingivalis* and *F. nucleatum* culture filtrates after a 48-h growth period was maintained between pH 7.0-7.4 while that of *P. nigrescens*, *P. intermedia*, and *A. actinomycetemcomitans* culture filtrates were between pH 5.5-6.0. Jurkat T cell apoptosis induction by culture filtrates was analyzed by colorimetric DNA fragmentation assay and gel electrophoresis. Cells cultured in medium supplemented with 10% of *P. gingivalis* culture filtrates showed a substantial increase in DNA fragmentation as compared with the control BHI-HMS broth (22-27% vs. 7%; Fig. 1a). This was further confirmed by gel electrophoresis as typical oligonucleosomal ladders indicative of fragmented DNA (Fig. 1b). *P. gingivalis* filtrates also significantly decreased
Jurkat T cells viability (67-78% vs. control; Fig. 1c). Likewise, *F. nucleatum* filtrates showed decreased Jurkat T cells viability (42% vs. control, Fig. 1c) and, in addition, induced Jurkat T cell DNA fragmentation (52% vs. 7% in the BHI-broth control, Fig. 1a and b). Interestingly, *Prevotella* spp. and *A. actinomycetemcomitans* culture filtrates failed to induce Jurkat T cell DNA fragmentation (5-9% vs. 7%) though they slightly decreased the viability of the cells (87-92% vs. control). SB, in the presence of 10% BHI-HMS (V/V) broth as a control to the filtrate groups, also decreased cell viability (79-94% vs. control) and increased the amount of Jurkat T cell DNA fragmentation (26-42% vs. 7% in the BHI-broth control) in a dose-dependent manner (Fig. 1a).

**P. gingivalis** and *F. nucleatum* culture filtrates induce apoptosis in monocytes/macrophages but not in fibroblasts and epithelial cells

Effects of the culture filtrates on apoptosis-dependent DNA fragmentation using human monocyte/macrophage cell lines THP-1 and U937 were determined. *P. gingivalis* and *F. nucleatum* culture filtrates significantly induced DNA fragmentation in both THP-1 (Fig. 2a and b) and U937 cells (Fig. 2c and d). By contrast, the *Prevotella* spp. culture filtrates did not exhibit any significant effect in both cell lines, while that of *A. actinomycetemcomitans* increased the amount of DNA fragmentation only in U937 cells (Fig. 2c). Moreover, SB-added BHI-HMS broth mixed with the cell cultures induced DNA fragmentation (Fig. 2a-d) and decreased cell viability (Fig. 2e) in both THP-1 and U937 cells as well as in lymphocytes. On the other hand, Ca9-22 oral epithelial cells (Fig. 3b) and N23 healthy gingival fibroblasts cells (Fig. 3d) did not exhibit typical laddering patterns when mixed with either culture filtrates or NaB. It is worth mentioning that a slight increase in DNA fragmentation was detected in N23 cells (Fig. 3c).

**SCFA-profile produced by periodontopathic bacteria**

As previously reported (4,5), it has been shown that SCFAs are major components produced by periodontopathic bacteria that suppress mitogenic activity and induce apoptosis in mouse and human lymphocytes. SCFA concentration was analyzed using the culture filtrates used for the apoptosis assays utilized in gas-liquid chromatography. Substantial amounts of butyric acid, which is the most effective volatile acid for inducing apoptosis of T cells, were detected in *P. gingivalis* (21.3-34.1 mM) and *F. nucleatum* (36.3 mM) culture filtrates. In contrast, trace levels (0.1-0.2 mM) of butyric acid were detected from *P. nigrescens*, *P. intermedia*, *A. actinomycetemcomitans* in...
cultural filtrates and the control BHI-HMS broth (Table 1). It is worth mentioning that propionic acid was detected at lower concentrations (4.7-9.5 mM) than butyric acid exclusively in the \textit{P. gingivalis} and \textit{F. nucleatum} culture supernatants. Likewise, iso-butyric acid (3.3-5.9 mM) and iso-valeric acid (10.3-17.0 mM) were detected in \textit{P. gingivalis} supernatants but not in \textit{F. nucleatum}.

### Discussion
Addition of \textit{P. gingivalis} and \textit{F. nucleatum} culture supernatants were seen to induce apoptosis of immune cells but not non-immune cells clearly showing that the representative periodontopathic bacteria, \textit{P. gingivalis} and \textit{F. nucleatum}, have deleterious effects on host immune cells both in acquired and innate immunity possibly through butyric acid. It has previously been reported that each spent medium from periodontopathic bacteria impairs T and B cell proliferation induced by mitogens, and volatile SCFAs in the medium are responsible for the inhibition of cell proliferation (4). Among SCFAs, butyric acid is a strong inducer of apoptosis and an inhibitor of cell proliferation and cytokine production of immune cells (4,5). This study clearly shows that butyric acid is a major component of SCFAs found in the \textit{P. gingivalis} and \textit{F. nucleatum} culture filtrates demonstrating its ability to induce apoptosis in immune cells (Table 1) and, likewise, further suggest that butyric acid is a major co-factor among SCFAs produced by periodontopathic bacteria responsible for apoptosis induction in immune cells.

In addition, the results of this study emphasize that propionic acid may also have partially contributed to apoptosis in this study, since it was detected in both \textit{P. gingivalis} and \textit{F. nucleatum} culture supernatants but not in \textit{Prevotella} spp. and \textit{A. actinomyces actinomycetemcomitans} (Table 1). Propionic acid has been reported to inhibit the proliferation of lymphocytes; however, the effect is considerably lower than butyric acid at 1-5 mM (4). Therefore, even though propionic acid may have partially contributed to apoptosis in immune cells, butyric acid is likely to be the key virulence factor secreted from these pathogens and is responsible for the apoptosis of host immune cells in periodontitis gingival tissue.

Peripheral blood monocytes can differentiate in vivo into both macrophages and dendritic cells (16,17). A low dose of butyrate (0.5 mM) partially inhibits the cytokine- or LPS-driven differentiation process of macrophages and dendritic cells from human monocytes (16,18,19) rather than inducing apoptosis. It has also been reported that in the presence of phorbol 12-myristate 13-acetate (PMA), a strong inducer of macrophage differentiation and maturation, 1 mM SB markedly induces apoptosis accompanied by impaired differentiation and that both differentiation-related and tumor necrosis factor-dependent mechanisms are involved in the potentiation of apoptosis by SB (20). Results obtained, however, showed that SB concentrations between 1.25-5.0 mM induced apoptosis even in undifferentiated monocytes. It maybe that butyric acid produced by periodontopathic bacteria could compromise the innate immune system in periodontal tissues by monocytic inducing apoptosis and inhibiting their differentiation to either macrophages or dendritic cells.

Interestingly, \textit{Prevotella} spp. and \textit{A. actinomyces actinomycetemcomitans} culture filtrates have little to no effect on apoptosis induction of Jurkat T cells and THP-1 monocytes, even though U937 monocytes displayed DNA

### Table 1 SCFAs production by periodontopathic bacteria

<table>
<thead>
<tr>
<th>Strain used for culture filtrate</th>
<th>SCFAs concentration (mM)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acetic</td>
<td>Propionic</td>
</tr>
<tr>
<td>----------------------------------</td>
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<td>----------</td>
</tr>
<tr>
<td>\textit{P. g} 33277</td>
<td>11.2 ± 0.4</td>
<td>4.7 ± 0.1</td>
</tr>
<tr>
<td>\textit{P. g} W83</td>
<td>14.8 ± 0.8</td>
<td>6.2 ± 0.1</td>
</tr>
<tr>
<td>\textit{P. g} W50</td>
<td>10.9 ± 0.6</td>
<td>6.2 ± 0.2</td>
</tr>
<tr>
<td>\textit{P. g} SU63</td>
<td>11.7 ± 0.4</td>
<td>9.5 ± 0.2</td>
</tr>
<tr>
<td>\textit{P. g} FDC381</td>
<td>10.2 ± 0.3</td>
<td>5.8 ± 0.2</td>
</tr>
<tr>
<td>\textit{P. n} 33563</td>
<td>15.3 ± 0.6</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>\textit{P. i} 25611</td>
<td>17.1 ± 1.0</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>\textit{F. n} 23726</td>
<td>10.9 ± 0.5</td>
<td>5.3 ± 0.2</td>
</tr>
<tr>
<td>\textit{A. a} Y4</td>
<td>17.1 ± 1.0</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>BHI-HMS broth</td>
<td>4.8</td>
<td>&lt; 0.4</td>
</tr>
</tbody>
</table>

SCFAs concentrations were measured in the BHI-HMS control broth or the 2-day culture filtrates of bacteria. Values are expressed as the mean ± SD of triplicate measurements except for the control broth. \textit{P. g}, \textit{P. gingivalis}; \textit{P. n}, \textit{P. nigrescens}; \textit{P. i}, \textit{P. intermedia}; \textit{F. n}, \textit{F. nucleatum}; \textit{A. a}, \textit{A. actinomyces actinomycetemcomitans}. 

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**Note:** The table and discussion content has been adapted from the original text for clarity and conciseness, while maintaining the scientific accuracy and context.
fragmentation. Among the virulence factors produced by these bacteria, leukotoxin, a 115-kDa protein, synthesized and released into the extracellular environment by A. actinomyctetcomitans exerts cytotoxic activity against human leukocytes (21). A. actinomyctetcomitans culture filtrate was found to be ineffective in apoptosis induction of lymphocytes suggesting that leukotoxin concentration was very low in the culture medium conditions used in this study. On the other hand, it has been reported that LPS from A. actinomyctetcomitans and P. gingivalis alone do not induce apoptosis of PMA-differentiated U937 cells (22). This would suggest that these virulence factors may not necessarily induce the apoptosis of immune cells and that butyric acid, which is not produced by both Prevotella spp. and A. actinomyctetcomitans strains tested in this study, can effectively induce apoptosis in the periodontal tissues of immune cells.

Although the molecular mechanisms behind the effects of SCFAs remain to be elucidated, it would seem that receptors, such as G-protein-coupled receptor (GPR) 43, contribute to the apoptosis of monocytes and lymphocytes induced by butyrate in the culture filtrates of periodontopathic bacteria. Free fatty acid receptors, such as GPR41 and GPR43, are functionally activated by SCFAs, including butyrate (23-26). GPR43 is highly expressed in hematopoietic tissues and immune cells, especially in monocytes, and is likely the main receptor contributing to the effect of SCFAs in immune cells (27).

It is worth mentioning that butyrate treatment of cells results in histone hyperacetylation inhibited a flurry of activity which led to the discovery that butyrate inhibits histone deacetylase (HDAC) activity (28,29). As previously reported, HDAC inhibitors such as butyrate could promote microbial infection (30,31). Although the role of HDAC inhibitors on immune cells has not been fully documented, it is possible that butyrate play an important role as a HDAC inhibitor in apoptosis induced by periodontopathic bacteria.

In conclusion, a soluble factor produced by the representative periodontopathic bacteria, P. gingivalis and F. nucleatum, induces apoptosis of immune cells in both innate and acquired immune systems. Butyric acid, a major component of SCFAs metabolized by these bacteria, is a key molecule able to exert a toxic effect. Inhibitory effect of butyrate in tissues could lead to the development of a novel strategy for the protection and treatment of periodontal diseases.

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