Apoptosis induced by Short-chain Fatty Acids Modulates Immunoresponses: Role of Cell-to-cell Communication in Inhibiting Butyric Acid-induced T Cell Apoptosis

Kuniyasu OCHIAI1* and Tomoko KURITA-OCHIAI2

1 Department of Bacteriology, Nihon University, School of Dentistry, 1-8-13, Kanda-Surugadai, Chiyoda-ku, Tokyo 101-8310, Japan
2 Department of Microbiology and Immunology, Nihon University, School of Dentistry at Matsudo, 2-870-1, Sakae-cho, Matsudo 271-8587, Japan

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Butyric acid present in the culture filtrates of Porphyromonas gingivalis, Prevotella loescheii and Fusobacterium nucleatum induced cytotoxicity and apoptosis in murine thymocytes, splenic T cells, and human Jurkat T cells. A pronounced accumulation of ROS occurred during butyric acid-induced apoptosis. Butyric acid induced apoptosis via the mitochondrial apoptotic pathway, e.g. cytochrome c, AIF, and Smac, and by the ceramide pathway. Up-regulation of JNK and p38, and down-regulation of ERK occurred immediately after butyric acid treatment. In microarray analysis, butyric acid treatment resulted in increased expression of the proapoptotic gene, whereas the expression of anti-apoptotic mediators was decreased. These data suggest that butyric acid is an apoptosis-inducing agent in most lymphoreticular cells. In contrast, epithelial cells and fibroblasts were insensitive to butyric acid. Fibroblasts from healthy gingival tissue rescued butyric acid-induced T cell apoptosis via proinflammatory cytokines such as IL-6 and IL-11, which were produced by the fibroblasts stimulated by butyric acid. Furthermore, the T cell apoptosis was also down-regulated by T cell adhesion to gingival fibroblasts through interaction with CD44, VLA-2, and VLA-5 expressed on T cells stimulated with butyric acid. Also, gingival fibroblasts from periodontal patients were highly susceptible to apoptosis induced by butyric acid when compared to healthy gingival fibroblasts. In conclusion, since short-chain fatty acids produced by periodontopathic bacteria induce apoptosis in immunological cells and the fibroblasts from periodontal patients, the results strongly suggested that they are concerned with the progress of periodontal disease.

Key words: apoptosis; short-chain fatty acids; periodontopathic bacteria; immunomodulation; cell-to-cell communication

Recently, it was reported in an extensive examination conducted in the United States that periodontal disease patients have higher susceptibility to arteriosclerosis, cardiac infarction and pneumonia (14). Furthermore, it was proved that periodontal disease was related to premature birth, a low weight of newborns and diabetes (14). These phenomena suggest that periodontopathic bacteria could be not only a source of focus infection, but also a reservoir of inflammatory materials. The mechanism of periodontal disease is very complicated and infection and its progress is related to the immunological imbalance.

It is recognized that periodontal diseases are infectious and that periodontal tissue breakdown results from the interaction of specific anaerobic bacteria, namely, Porphyromonas, Prevotella, and Fusobacterium spp., and host immune mechanisms. These bacteria produce an elaborate variety of virulence factors such as proteases, lipopolysaccharides (LPS), and fimbriae. Considering the evidence that some periodontopathic bacteria cause bacterial endocarditis in extra-oral sites, we hypothesized that immunosuppressive substances are involved as causes of the diseases. Therefore, we purified the immunosuppressive factors and characterized their properties (3, 11). We could not detect immunosuppressive substances in sonic extracts from P. gingivalis (10), so we examined the effects of extracellular metabolites from periodontopathic bacteria on the proliferation of and cytokine production of mouse splenic cells as a potential mechanism of imbalance among host-microbial interactions.

The respective metabolisms of these bacteria are also characterized by the production of an identifiable fingerprint of short-chain fatty acids, which are major by-products of anaerobic metabolism released into the microenvironment at the infection site, and can diffuse
across biological membranes. Previous studies have demonstrated that these fatty acids exert inhibitory effects on gingival fibroblast proliferation, and phagocytosis. These findings are consistent with the pathogenic role of these molecules in periodontal diseases (13).

In the present study, we demonstrated that short-chain fatty acids (SCFA), especially butyric acid present in the culture filtrates of *P. gingivalis*, *P. loescheii*, and *F. nucleatum*, greatly inhibit murine T and B cell proliferation and cytokine production (2). This evidence clearly supports the hypothesis that activation of apoptosis is at least one essential step in the butyric acid-induced immunosuppressive pathway and that butyric acid can modulate the immunoregulatory cell population in peripheral tissues by inducing B and T cell death and gingival fibroblast death from periodontal disease through apoptosis.

**VOLATILE FATTY ACIDS PRODUCED BY PERIODONTOPATHIC BACTERIA**

A low molecular weight, heat-stable agent present in the two-day culture filtrate of *P. gingivalis*, *P. loescheii*, and *F. nucleatum* significantly depressed Con A- and LPS- induced cell proliferation (2). To determine whether SCFA present in the filtrate could account for this depression, we tested extracted volatile fatty acids (VFA) and non-VFA for their effects on mitogenic activity. The VFAs extracted from immunsuppressive supernatants greatly inhibited T and B cell proliferation. Among these VFAs, butyric, propionic, valeric, and isovaleric acids impaired cell proliferation dose-dependently (2). Gas-liquid chromatographic analysis data suggested that immuno-inhibitory activities in culture filtrates were mainly attributable to butyric and isovaleric acids in *P. gingivalis*, to propionic, butyric, and isovaleric acids in *P. loescheii*, and to butyric acid in *F. nucleatum*. The following graded activity series was evident: butyric > isovaleric > propionic acids. Especially butyric acid strongly suppressed T and B cell proliferation (2). Our results showed that from 13.3 to 26.8 mM of butyric acid was detected in culture filtrates of *P. gingivalis*, *P. loescheii* and *F. nucleatum*, and a previous study showed that butyric acid concentrations in subgingival plaque from periodontitis sites could reach 14.4 mM (9). Thus, butyric acid can be recognized as the most important virulent factor in SCFA of periodontopathic bacteria. However, isovaleric acid, which is common to *P. gingivalis*, and *P. loescheii*, and propionic acid, which are major products of *P. loescheii*, also significantly inhibited cell proliferation at 10 mM.

*P. gingivalis*, *P. loescheii* and *F. nucleatum* also produce indole and ammonia and volatile sulfur compounds including hydrogen sulfide, dimethylsulfide, and methylmercaptan, all of which are potentially cytotoxic factors. These toxic factors could have been present in the spent media and may be responsible for some of the inhibitory effects of mitogen activity and cytokine production. However, since most of these are heat-labile, they would seem to contribute little to the inhibitory effect; i.e. the substance which inhibits mitogenic activity is heat-stable.

**BUTYRIC ACID-INDUCED APOPTOSIS DEPENDS ON GENERATION OF REACTIVE OXYGEN SPECIES**

We have previously demonstrated that butyric acid induces cytotoxicity and apoptosis of murine thymocytes, splenic T cells, and human Jurkat T cells (4). Butyric acid-treated cells showed higher basal levels of reactive oxygen species (ROS) compared with untreated cells (6). Furthermore, antioxidants NAC and 3AB showed a protective effect against butyric acid-induced apoptosis in a dose-dependent manner. This data provides evidence that the generation of ROS is essential for the induction of apoptosis by butyric acid. Our previous study demonstrated that caspase-8 and -9, as well as caspase-3-dependent apoptosis plays an important role in butyric acid-induced T cell death. Caspase-3, -9, and especially -8 activities were dose-dependently reduced by pretreatment with NAC. These results indicate that ROS production plays a role as a regulator of caspase activation in butyric acid-induced apoptosis (6).

**Effect of mitochondria, ceramide, and MAP kinase in butyric acid-induced apoptosis**

Recent evidence indicates that mitochondria play a pivotal role in apoptosis in multicellular organisms by releasing apoptotic factors such as cytochrome c, Smac, and AIF. Because mitochondria play an important role in many forms of apoptosis, we investigated the involvement of mitochondria as the source of ROS. Cytosolic levels of AIF and Smac were detected immediately and increased throughout the 3 h period after butyric acid treatment. These data suggest that butyric acid induces apoptosis via the mitochondrial apoptotic pathway. Exposure of cells to butyric acid increased cellular ceramide in a time-dependent manner. We used DHS, which is a potent inhibitor of sphingosine kinase, to examine the effect of kinase inhibition on ceramide mediated by DHS in a dose-dependent manner.
The results suggested the involvement of SPP in ceramide-mediated T cell apoptosis by butyric acid. We examined the influence of butyric acid on JNK, p38, and ERK phosphorylation. The levels of JNK phosphorylation had increased markedly by 0.5 h after the start of treatment with butyric acid. A sustained phosphorylation was down-regulated after 6 h following treatment with butyric acid. Thus, butyric acid activates the JNK and p38 pathways and at the same time down-regulates ERK in Jurkat cells (6).

Microarray analysis of Jurkat cells

To examine the transcriptional pathways activated downstream of butyric acid sensitization, we used a cDNA microarray to monitor transcriptional changes in Jurkat cells treated with butyric acid. Butyric acid treatment primarily resulted in increased expression of proapoptotic genes such as Bax, Bad, Bak, and caspase-3, -6, -7, -8, and -9, whereas the expression of anti-apoptotic mediators such as Bcl-2 and glutathione was decreased (Table 1). Furthermore, among the MAPKs, the repression profile of butyric acid-treated Jurkat cells was confirmed by means of cDNA array (6).

Table 1. Differential gene expression by DNA microarray analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Change in expression (fold)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7 h</td>
<td>17 h</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>-2.6</td>
<td>-3.6</td>
</tr>
<tr>
<td>BAK</td>
<td>2.8</td>
<td>2.5</td>
</tr>
<tr>
<td>BAX</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>BAD</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>ERK1</td>
<td>-1.2</td>
<td>-1.7</td>
</tr>
<tr>
<td>JNK3A2</td>
<td>1.6</td>
<td>2.1</td>
</tr>
<tr>
<td>Glutathione</td>
<td>-1.9</td>
<td>-3.7</td>
</tr>
<tr>
<td></td>
<td>-3.6</td>
<td>-2.4</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>Caspase 6</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>Caspase 7</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>Caspase 8</td>
<td>2.4</td>
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</tr>
<tr>
<td>Caspase 9</td>
<td>1.6</td>
<td>1.9</td>
</tr>
<tr>
<td>CRAF1</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>DAPK1</td>
<td>-1.3</td>
<td>-1.5</td>
</tr>
<tr>
<td>NFKB</td>
<td>-1.4</td>
<td>-3.0</td>
</tr>
</tbody>
</table>

Jurkat cells were treated for the indicated times with or without 5 mM butyric acid. Total RNA was isolated, retrotranscribed, 32P-labeled and hybridized to the cDNA array representing 205 transcripts of known genes. The signals were then analyzed by NIH Image 1.61 software and expressed as fold of increase with respect to the untreated cells. A positive number indicates that a gene was expressed at higher levels in butyric acid-treated Jurkat cells than in untreated Jurkat cells, and a negative value indicates higher expression in untreated Jurkat cells.

Coculturing Jurkat cells with fibroblasts in pore-filled culture inserts attenuated butyric acid-induced apoptosis. Since IL-8 slightly stimulated, and IL-6 and IL-11 significantly suppressed butyric acid-induced Jurkat cell apoptosis in a dose-dependent manner, our results suggest that the attenuation of butyric acid-induced T cell apoptosis by gingival fibroblast is the result of the effects of proinflammatory cytokines, such as IL-6 and IL-11, which are produced in fibroblasts stimulated by butyric acid (7).

Fibroblasts rescue T cell apoptosis via cell-to-cell contact

We also determined if T cell adhesion to gingival fibroblasts influenced the susceptibility of T cells to butyric acid-induced apoptosis. The number of T cells adhering to fibroblasts was significantly increased by the addition of butyric acid. All T cells adhering to fibroblasts remained viable, while the inadherent cells underwent apoptosis. The increase in T cell adhesion to fibroblasts was also observed when T cells, but not fibroblasts, were pretreated with butyric acid. CD44, VLA-2, and VLA-5 expressions on T cells were increased following treatment with monoclonal antibodies against CD44, VLA-2 and VLA-5 followed by co-culture with fibroblasts and abolished T cell adhesion to fibroblasts (8). These results indicate that the T cell adherence to fibroblasts is enhanced by butyric acid.
acid, and that butyric acid-induced T cell apoptosis is down regulated by T cell adhesion to fibroblasts (Fig. 1).

**Lymphoreticular cells are sensitive to butyric acid**

The results of DNA fragmentation assays indicate that butyric acid rapidly induces apoptosis in thymocytes and in splenic T cells and Jurkat cells (4). Similar results were obtained by using B cell lines, such as Raji and WEHI 231 cells (5). However, epithelial cells and fibroblasts were not sensitive (12). These data suggest that butyric acid is an apoptosis-inducing agent in most lymphoreticular cells.

**Butyric acid induces apoptosis in gingival fibroblasts isolated from patients with adult periodontitis**

Butyric acid significantly suppressed the viability of inflamed gingival fibroblasts (IGF) and induced apoptosis in a concentration-dependent manner. Incubation of IGF with butyric acid resulted in the typical ladder pattern of DNA fragmentation, chromatin condensation and hypodiploid nuclei and mitochondrial injury. Furthermore, butyric acid-induced apoptosis in IGF was partially reduced by the inhibitors for initiator caspases (caspase-8 and -9) and effector caspases (caspase-3, -6, and -7). These results suggest that butyric acid can highly induce fibroblast apoptosis followed by destruction of gingival tissues in inflamed periodontal lesions.

**CONCLUSION**

We have illustrated our results in a schematic diagram of apoptosis induced by butyric acid, a metabolic by-products of periodontopathic bacteria (Fig. 1). Emerging evidence indicates that bacterial modulation of apoptosis is an important part of pathogenesis (1). Specific pathogens or their extracellular products may directly induce apoptosis (15) and will promote bacterial invasion at the sites of infection.

Our results clearly indicate that interactions between butyric acid-pretreated gingival fibroblasts and T cells prevented butyric acid- or Fas-induced T cell apoptosis. The proinflammatory cytokines such as IL-6 and IL-11 produced in fibroblasts stimulated by butyric acid, and the interaction of CD44, VLA-2, and VLA-5 expressed on T cells stimulated by butyric acid with their ligands, were involved in the attenuation of T cell apoptosis by gingival fibroblasts. These results suggest that fibroblast could rescue butyric acid-induced T cell apoptosis in healthy gingival tissue.

Furthermore, inflamed gingival fibroblasts from adult periodontitis patients are highly susceptible to apoptosis induced by butyric acid when compared to healthy gingival fibroblasts. These results suggest that butyric acid-induced apoptosis is associated with the destruction of the supporting tissues of the tooth.
REFERENCES