Differences in Cytokine Gene Expression after a Stimulation with \textit{Escherichia Coli} and \textit{Porphyromonas Gingivalis} or Lipopolysaccharides Derived from these Bacteria

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Abstract

Monocytes are important cells in innate immunity. The early stage of the innate immunity is regulated by various cytokines produced by monocytes. We conducted a preliminary study to investigate TNF\textalpha expression by stimulating THP-1 cells with several bacterial species. The TNF\textalpha mRNA levels significantly varied, with the most potent stimulatory effects observed with \textit{P. gingivalis}. In the present study, we focused on \textit{P. gingivalis} and compared differences in cytokine expression profiles after the stimulation of THP-1 with \textit{E. coli}. Bacterial antigen stimulation increased various cytokine gene expressions in THP-1. \textit{P. gingivalis} had significantly more potent effects on the mRNA expressions of TNF\textalpha, IL-1\beta, and IL-10, but not of IL-12p40, than \textit{E. coli}. This result suggests the potential ability of \textit{P. gingivalis} to induce inflammation. THP-1 stimulated with LPS derived from both bacterial species showed that \textit{E. coli} had significantly more potent effects on the expressions of TNF\textalpha, IL-1\beta, and IL-12p40 than \textit{P. gingivalis}. The differences in the bacterial antigens and the LPS stimulation effects suggest involvements of different receptors, such as TLR-2 and -4, which recognize bacterial components. The present results suggest that the \textit{P. gingivalis} somatic cell antigen stimulates a number of pattern recognition receptors at the same time as the synthesis of bacterial components, except LPS. The potent virulence of \textit{P. gingivalis} and persistence of infection might be affected by differences in cytokine production. Pro-inflammatory responses are dependent not only on the bacterial type, but also bacterial components.

Keywords: Porphyromonas gingivalis (P. gingivalis), Escherichia coli (E. coli), Lipopolysaccharide (LPS), Toll-like receptors (TLR), Cytokines, Inflammation.

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1. Introduction

The innate immune system mostly comprises circulating monocytes that recognize conserved bacteria-associated molecules, such as lipopolysaccharide (LPS) and peptidoglycan (PGN), and differentiate into macrophages in inflamed tissues. These cells express toll-like receptors (TLR) which play a central role in microbial molecular pattern detection. TLR function in signaling pathways via a common adaptor, MyD88, and in the activation of transcription factors, such as nuclear factor kappa B (NF-κB). Important pro-inflammatory cytokines secreted by macrophages in response to bacterial products through TLR include tumor necrosis factor α (TNFα), interleukin (IL)-1β, IL-6, and IL-12. TNFα and IL-1β are inducers of local inflammatory responses that help to contain infections. In patients with severe sepsis, the host reaction may be characterized as an excessive inflammatory response caused by a cascade of TNFα and IL-1β. It is important to elucidate cytokine expression profiles in order to identify the pathological condition of infectious disease.

Bacterial infections are mediated in part by the production of a number of inflammatory cytokines; however, the pattern of early cytokine production may be dependent on the specificities of the microbial pathogens. Beran et al. suggested higher productivity of cytokines with gram-negative bacteria than with gram-positive bacteria. LPS is a Gram-negative bacterial endotoxin, while PGN is a Gram-positive bacterial cell wall component that mainly exerts its effects by activating monocytes and macrophages. However, the mechanisms that trigger the different activation patterns of cytokine profiles in these cells remain unclear. We hypothesize that the reactivity of innate immune cells might vary with bacterial strains, and might trigger different inflammatory responses that are dependent on the microbial constituents of bacterial cell walls.

Porphyromonas gingivalis (P. gingivalis) is a Gram-negative rod-shaped bacterium and a key periodontal pathogen associated with the etiology of periodontal disease. Periodontal disease has been linked to systemic disorders, such as atherosclerosis and type 2 diabetes. The pathogenic components of P. gingivalis-LPS is one of the main factors associated with periodontitis and P. gingivalis-LPS would trigger various inflammatory responses. Escherichia coli (E. coli) is a Gram-negative rod-shaped bacterium with LPS that has the ability to stimulate monocytes and macrophages. In the present study, we compared differences in inflammatory cytokine expression profiles after the stimulation of THP-1 human mononcytic cells with E. coli, P. gingivalis, and LPS derived from these bacteria.

2. Materials and Methods

2.1. Bacterial Strains and LPS

All bacterial strains were transferred from Clinical Laboratory, Chiba Emergency Medical Center, Chiba, Japan. Clinically isolated P. gingivalis was grown on pre-reduced Brucella broth agar from Kyokuto Pharmaceutical Industrial (Tokyo, Japan), and incubated at 37°C for 48-72 h in an anaerobic conditioning chamber. E. coli (DX37t), Klebsiella pneumoniae (clinically isolated strain), Haemophilus influenzae (ATCC 49619), Staphylococcus aureus (ATCC 29213), and Streptococcus pneumoniae (ATCC 49619) were grown on LB broth from Kyokuto Pharmaceutical Industrial (Tokyo, Japan), and incubated at 37°C for 48-72 h in an anaerobic incubator. Each bacterial strain was killed in 95°C heated water and then treated with 0.5% formalin. Fixed bacteria were washed twice with phosphate-buffered saline (PBS). Bacterial concentrations were adjusted by the McFarland standards method for 1.5 unit (OD600: 0.354), namely, approximately 100 µg/mL. Standard LPS from P. gingivalis was purchased from Invitrogen (San Diego, CA, USA). Standard LPS from E. coli O26 was purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Cell Culture and Stimulation

Human monocyctic THP-1 cells were obtained from the Cell Engineering Division, RIKEN BioResource Research Center (Tsukuba, Ibaraki, Japan). Cells were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS), L-glutamine (2 mM), 1% Antibiotic/Antimycotic solution (Sigma-Aldrich, St. Louis, MO, USA), and maintained at 37°C in a humidified 5% CO2 incubator. Cultured cells after 2-3 passages were designated as appropriate for subsequent experiments. Cells were placed into 24-well plates at a concentration of 2 × 104 cells/mL/well in conditioned media. Each regent was added to the culture solution and incubated for the indicated time. Regent concentrations were as follows: E. coli and P. gingivalis-cell antigen: 10 µg/mL; E. coli and P. gingivalis-LPS: 1 µg/mL. Cells treated with PBS were basal controls.

2.3. RNA Extraction and Reverse Transcription

Total RNA was isolated from THP-1 cells using the acid guanidinium-phenol-chloroform method regent, ISOGEN (Nippon Gene, Tokyo, Japan), according to the manufacturer’s instructions and then eluted in RNase-free water. Total RNA was quantified using the spectrophotometer NanoDrop (Thermo Fisher Scientific, MA, USA).

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and total RNA (1 μg) was then reverse transcribed using random hexamers in 10 μL reactions with the cDNA synthesis kit Takara Prime Script RT Master Mix (Takara Bio, Kusatsu, Shiga, Japan), according to the manufacturer’s instructions.

2.4. Cytokine Gene Expression Assay

Quantitative reverse transcriptase (RT)-PCR was performed using the StepOne Real-Time PCR system (Thermo Fisher Scientific, MA, USA) in at least three separate experiments. Amplification reactions were performed in a final volume of 20 μL containing 10 μL of SYBR Premix Ex Taq (Takara Bio, Kusatsu, Shiga, Japan), 1 μL of the cDNA template, and 0.8 μM of each pair of primers. The target cytokine genes to be compared were TNFa, IL-1β, IL-6, IL-10, IL-12p10, and TGF-β. The expression of the endogenous gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The expression level of each cytokine gene was normalized to GAPDH (threshold cycle value: Ct) and fold changes for each gene were calculated by comparing the stimulated test and untreated controls from Ct values according to the Ct comparative ΔΔCt method. Real-time PCR primer pairs are described in Table 1.

Table 1. Primer pairs for the quantification of gene expression using a real-time reverse transcription-Polymerase Chain Reaction (RTPCR).

<table>
<thead>
<tr>
<th>Targets</th>
<th>Sequence (5'-3')</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH-F</td>
<td>GCACGCGTCAAGGGCTGAGAAC</td>
<td>138 bp</td>
</tr>
<tr>
<td>GAPDH-R</td>
<td>TGGTGAAGACCCAGTGGGA</td>
<td>138 bp</td>
</tr>
<tr>
<td>TNFα-F</td>
<td>GAACACTGGAGATCTCAGAATG</td>
<td>246 bp</td>
</tr>
<tr>
<td>TNFα-R</td>
<td>GTCTCAAGGAAGTCGATGAGAAC</td>
<td>246 bp</td>
</tr>
<tr>
<td>IL-1β-F</td>
<td>GACACAGGGGATAACGAGCC</td>
<td>248 bp</td>
</tr>
<tr>
<td>IL-1β-R</td>
<td>AGCGACAGGAGGTACAGATT</td>
<td>248 bp</td>
</tr>
<tr>
<td>IL-10-F</td>
<td>ACAGCTCGACAGTCGCTG</td>
<td>329 bp</td>
</tr>
<tr>
<td>IL-10R</td>
<td>AGTTCACATCGCCCTGATG</td>
<td>329 bp</td>
</tr>
<tr>
<td>IL-12p10-F</td>
<td>CTTAGGCTCTGGCAAAACCT</td>
<td>102 bp</td>
</tr>
<tr>
<td>IL-12p10-R</td>
<td>AGGAGGGAATGGCTTGAAGACC</td>
<td>102 bp</td>
</tr>
<tr>
<td>IL-6-F</td>
<td>TCTCCACAAGGGGCTTGC</td>
<td>193 bp</td>
</tr>
<tr>
<td>IL-6-R</td>
<td>CTCAGGCGCTGAAGTCCC</td>
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</tr>
<tr>
<td>TGFβ-F</td>
<td>GCCCTGGACACCAACTTTG</td>
<td>129 bp</td>
</tr>
<tr>
<td>TGFβ-R</td>
<td>GAAGTTGCGATGGTACCGTCC</td>
<td>129 bp</td>
</tr>
</tbody>
</table>

2.5. Statistical Analysis

Data were showed as means ± standard error (SE). A two- or three-way analysis of variance (ANOVA) was used to analyze differences among multiple groups. Differences between groups were considered to be significant when the P value was < 0.05.

3. Results

In the preliminary experiment, changes in the gene expression level of the pro-inflammatory cytokine TNFa were examined with 1 μg/mL of various bacteria somatic cell antigens in THP-1 cells (8-hour stimulation). Figure 1 shows significant differences in TNFa mRNA expression levels according to the species of bacteria (P< 0.05). The stimulus with P. gingivalis was stronger than those with the other bacteria.

The temporal early inflammatory gene expression pattern was evaluated in THP-1 cells following treatment with formalin treated E. coli- or P. gingivalis-somatic cell antigen by quantitative RT-PCR. An inflammatory response was indicated by significant increases in the mRNA levels of TNFa, IL-1β, IL-12p10, and IL-10 from the basal value (P< 0.01). The results obtained were shown in Figure 2a ~ 2d, and indicated that the gene expression levels of TNFa, IL-1β, and IL-10 in THP-1 cells were significantly higher with P. gingivalis than with E. coli (P< 0.01). Regarding IL-12p10 expression levels in THP-1 cells, the P. gingivalis somatic cell antigen stimulus was weaker than that of E. coli. No significant changes were observed in the responses of IL-6 and TGF-β levels to both bacteria for at least 6 hours after the stimulation, while a modest change (16-fold) was noted in the P. gingivalis (but not E. coli)-induced expression of IL-6 24 hours after the stimulation (data not shown). Overall, the ability of P. gingivalis to stimulate somatic cells was more potent than that of E. coli.

In contrast, differences were observed in the results obtained using the LPS stimulation by E. coli or P. gingivalis and somatic cell antigen stimulation patterns. The results obtained were shown in Figure 3a ~ 3d and indicated that the up-regulated expression of TNFa, IL-1β, and IL-12p10 was significantly stronger with LPS from E. coli than with that from P. gingivalis (P< 0.01). The stimulatory ability of E. coli-LPS was stronger than that of P. gingivalis-LPS. However, no significant changes were observed in IL-10 expression levels following either LPS stimulus.

When time courses of gene expression were investigated from E. coli- and P. gingivalis-somatic cell antigens for 24 hours after the stimulation Figure 4a and 4b, the maximum level of TNFa expression by E. coli was noted 4 hours after the stimulation, whereas the maximum level of P. gingivalis was 2 hours. The maximum expression levels of IL-1β and IL-10 were at the same time point, 8 hours after the stimulation with E. coli- and P. gingivalis- somatic cell antigens. The approximately 250-fold reduction in IL-10 expression levels after the E. coli stimulation persisted for 24 hours, whereas a decrease to approximately 40-fold was noted with the P. gingivalis stimulation. The maximum level of IL-12p10 expression by P. gingivalis was noted 12 hours after the stimulation, whereas the level of IL-
12p40 by E. coli continued to increase even after 24 hours. The increase and decrease patterns and the expression levels were significantly different between E. coli and P. gingivalis ($P < 0.01$).

Figure 1. Preliminary analysis for TNFα gene expression changes in THP-1 cells by various bacteria. THP-1 cells were stimulated with 1 μg/mL of the indicated bacteria somatic cell antigen or vehicle (phosphate-buffered saline: PBS) for 8 hours. The results obtained were normalized to GAPDH and calculated as a fold change from basal levels using quantitative real-time RT-PCR. P. gingivalis was stronger than those with the other pathogens. Significant differences between various bacteria groups by Dunn method of multiple comparison ($P < 0.05$).

Source: PowerPoint2016.

(a) Source: PowerPoint2016.

(b) Source: PowerPoint2016.
Figure 2. Cytokine gene expression (mRNA) changes by \textit{E. coli}- and \textit{P. gingivalis}-somatic cell antigens in THP-1 cells 2, 4, and 6 hours after the stimulation. 10 μg/mL \textit{E. coli} and 10 μg/mL \textit{P. gingivalis} up-regulate TNFα \textit{(a)}, IL-1β \textit{(b)}, IL-12p40 \textit{(c)}, and IL-10 \textit{(d)}. Data are expressed as means ± SE. Results were normalized to GAPDH and calculated as a fold change from basal levels using quantitative real-time RT-PCR.

Note: Significant differences between \textit{E. coli}- and \textit{P. gingivalis}-somatic cell antigen groups by a two-way ANOVA \textit{(P < 0.01)}. 

Source: PowerPoint2016.
Figure-3. Cytokine gene expression (mRNA) changes by E. coli- and P. gingivalis-LPS in THP-1 cells 2, 4, and 6 hours after the stimulation. 1 μg/mL E. coli-LPS and 1 μg/mL P. gingivalis-LPS up-regulate TNFα (a), IL-1β (b), IL-12p40 (c), and IL-10 (d). Data are expressed as means ± SE. Results were normalized to GAPDH and calculated as a fold change from basal levels using quantitative real-time RT-PCR. Significant differences between E. coli- and P. gingivalis-LPS groups by a two-way ANOVA (P < 0.01) except to IL-10. NS: no significant differences. ND: not detected.

4. Discussion

Innate immunity is a system related to the first biophylaxis to infection by pathogens. Monocytes assume a role that is important for immune responses. They possess pattern recognition receptors that bind pathogen structures, such as TLR, receive pathogen infection information, and activate the initial inflammatory response. Activated monocytes differentiate to macrophages and produce inflammatory cytokines, and also play an important role in acquired immunity. Factors related to inflammatory cytokine production depend on pathogen compositions and interactions with pattern recognition receptors. Differences in the types of pathogens lead to variations in cytokine production and profiles. Our preliminary study showed that differences in TNFα mRNA expression levels according to the species of bacteria were more significant than the distinction of Gram-negative and -positive bacteria. Therefore, we compared the effects of P. gingivalis and E. coli on TNFα expression levels.

The expression levels of various types of cytokine genes increased in THP-1 cells stimulated with formalin-treated bacteria. Regarding their effects on the mRNA expression of TNFα, IL-1β, and IL-10, P. gingivalis was clearly stronger than E. coli. This result suggests the potenti ability of P. gingivalis to induce inflammation, which may be a factor contributing to the progression of periodontal disease and the onset of general clinical conditions. Interestingly, this was not the case for IL-12p40. However, E. coli showed significantly more potent and lasting bacterial and LPS stimulation on IL-12 p40 than P. gingivalis. IL-12 stimulates interferon (INF)γ production and the differentiation of Type 1 T-helper cells and functions to induce acquired immunity [13]. P. gingivalis may contribute to persistent survival by suppressing the immune mechanisms of the host more weakly than E. coli. The expression of anti-inflammatory cytokine IL-10 was significantly reduced 24 hours after stimulation with P. gingivalis, unlike with E. coli. Differences in the IL-12p40 and IL-10 expression time course may play a role in the sustained viability of P. gingivalis. On the other hand, TNFα, IL-1β, and IL-12p40 expression levels were increased by the stimulation with LPS derived from both bacteria. However, the effects of E. coli-LPS were stronger than those of P. gingivalis-LPS, and this pattern was opposite to that of the bacterial cell stimulation in either case. Moreover, the increased expression of IL-10 was not confirmed at least 6 hours after the stimulation. Barsby et al. reported the increased production of TNFα, IL-1β, the IL-18 receptor and IL-6 in THP-1 cells stimulated with E. coli-LPA and P. gingivalis-LPS [14].
As observed in our study, E. coli-LPS had more potent effects than P. gingivalis-LPS. However, the increased expression of IL-6 was not observed in the present study. Su et al. stimulated rat bone marrow-induced dendritic cells with E. coli-LPS and P. gingivalis-LPS, and reported that the production of IL-12 was enhanced more by E. coli-LPS, while that of IL-10 was greater with P. gingivalis-LPS [18]. Some cytokine expression profiles were inconsistent between previous findings and the present results, and this may be attributed to differences in the origin strains of LPS used in experiments. In other words, in addition to differences in strains, differences in the molecular structures of bacterial components appear to have subtly influenced innate immune cells. The results obtained in the present study showed that P. gingivalis bacterial cells induced immune responses that were stronger than those caused by purified LPS. Barksby et al. [14] and Su et al. [15] reported that the different effects between E. coli- and P. gingivalis-LPSs were caused by the involvement of TLR-4 or -2. MyD88 is commonly involved in the signaling pathways of various TLRs. However, there are MyD88-independent pathways, and Toll/IL-1 receptor (TRI) domain-containing adapters, TRIF, is involved in the TLR-4 pathway, but not in the TLR-2 pathway [16]. E. coli-LPS activates both pathways of MyD88 and TRIF as an agonist of TLR-4, while P. gingivalis-LPS is not involved in the TRIF pathway because it is an agonist of TLR-2. Therefore, E. coli-LPS had more potent effects. However, the P. gingivalis somatic cell antigen has been suggested to stimulate various types of pattern recognition receptors at the same time as the synthesis of bacterial components, except for LPS, while it causes a strong response.

P. gingivalis is the most important pathogen involved in the onset of chronic periodontal diseases and also plays a role in systemic clinical conditions. Omura et al. reported a case of death by sepsis due to P. gingivalis [17]. These findings demonstrate that P. gingivalis causes strong systemic inflammation as well as periodontal diseases. The present results confirmed that P. gingivalis induced inflammation more strongly than other pathogens. Differences in the types of pathogens affect not only various stimuli but also various properties. This might influence the difference in pathogenicity. Sun et al. reported inhibited TNFα expression and enhanced IL-10 expression in a study on endotoxin tolerance (the suppression of some inflammatory responses) by repeated exposure to LPS [18]. In their study, the endotoxin tolerance-inducing effect by P. gingivalis-LPS was more suppressive than that of E. coli-LPS. As described above, we confirmed that the IL-10 expression time course after the P. gingivalis bacterial cell stimulation disappeared faster than that after the E. coli bacterial cell stimulation. A difference in the expression profile of the anti-inflammatory cytokine IL-10 may contribute to this complex in vivo inflammatory response. A number of phenomena have not been clarified in the innate immune system yet. In the future, we will investigate the pathology of endotoxin tolerance associated with treatment resistance.

References


