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Streptococcus gordonii lipoproteins induce IL-8 in human periodontal ligament cells

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ABSTRACT

Streptococcus gordonii, a Gram-positive oral bacterium, is a life-threatening pathogen that causes infective endocarditis. It is frequently isolated from the periapical lesions of patients with apical periodontitis and has thus been implicated in inflammatory responses. However, little is known about the virulence factors of *S. gordonii* responsible for the induction of inflammatory responses in the periapical areas. Here, we investigated the role of *S. gordonii* cell wall-associated virulence factors on interleukin (IL)-8 induction in human periodontal ligament (PDL) cells using ethanol-inactivated wild-type *S. gordonii*, a lipoteichoic acid (LTA)-deficient mutant (Δ*ltaS*), and a lipoprotein-deficient mutant (Δ*lgt*). Wild-type *S. gordonii* induced IL-8 expression at both the protein and mRNA levels in human PDL cells in a dose- and time-dependent manner. A transient transfection and reporter gene assay demonstrated that wild-type *S. gordonii* activated Toll-like receptor 2 (TLR2). Additionally, IL-8 production induced by wild-type *S. gordonii* was substantially inhibited by anti-TLR2-neutralizing antibodies. Both wild-type *S. gordonii* and the Δ*ltaS* mutant induced IL-8 production; however, this response was not observed when cells were stimulated with the Δ*lgt* mutant. Interestingly, lipoproteins purified from *S. gordonii* induced IL-8 production, whereas purified LTA did not. In addition, purified lipoproteins stimulated TLR2 more potently than LTA. Furthermore, *S. gordonii*-induced IL-8 expression was specifically inhibited by blocking p38 kinase, while lipoprotein-induced IL-8 expression was inhibited by blocking p38 kinase, ERK, or JNK. Of particular note, exogenous addition of purified *S. gordonii* lipoproteins enhanced Δ*lgt*-induced IL-8 production in human PDL cells to an extent similar to that induced by the wild-type strain. Collectively, these results suggest that lipoproteins are an important component of *S. gordonii* for the induction of IL-8 production in human PDL cells through TLR2 activation. Therefore, lipoproteins potentially contribute to inflammatory apical periodontitis.

1. Introduction

Apical periodontitis is an inflammatory disease in the apical region that is a result of endodontic infection by bacteria residing in the apical root canal (Nair, 2004). These bacteria can enter the apical periodontal ligament through the apical foramen, thereby causing local inflammation, destruction of periapical tissues, and resorption of alveolar bone in the lesion (Nair, 2004; Silva et al., 2007). During apical periodontitis caused by bacterial infection, chemokines contribute to local inflammation by inducing heavy infiltration of immune cells (Silva et al., 2007). In particular, interleukin (IL)-8 has been detected at a high level in most exudates from infected root canals with periapical lesions (Marton et al., 2000; Silva et al., 2007). This cytokine causes extensive infiltration of neutrophils, which play an important role in inflammation (Nair, 2004; Yoshimura et al., 1987). Additionally, IL-8 can recruit and activate osteoclasts at the infection site, leading to resorption of hard tissues related to apical abscess (Bendre et al., 2003; Silva et al., 2007). Furthermore, IL-8 level in periapical lesions has been shown to be correlated with pain in patients with apical periodontitis (Shimauchi et al., 2001).

Apical periodontitis and refractory apical periodontitis are associated with a variety of bacteria including *Streptococcus gordonii*,

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Enterococcus faecalis, Fusobacterium nucleatum, and *Tannerella forsythia* (Rocas et al., 2008; Rocas and Siqueira, 2012). *S. gordonii*, a Gram-positive facultative anaerobic bacterium, is found in the human oral microflora (Colombo et al., 2009). *S. gordonii* is a particularly harmful pathogen that causes infective endocarditis after it enters the blood (Xiong et al., 2008). In addition, *S. gordonii* has been associated with dental infective disease such as dental caries and oral periodontitis (Chavez De Paz et al., 2005; Svensater et al., 2003). *S. gordonii* is frequently found at and isolated from periapical lesions after treatment of apical periodontitis (Chavez de Paz et al., 2005) because it can form biofilms on the root canal surface (Love and Jenkinson, 2002). Moreover, *S. gordonii* can exchange virulence genes encoding cytotoxins, adhesins, and antibiotic resistance genes with *E. faecalis* (Sedgley et al., 2008). Thus, *S. gordonii* is intimately involved in apical periodontitis and refractory apical periodontitis. However, despite its importance, the pathogenic mechanism by which *S. gordonii* contributes to apical periodontitis and refractory apical periodontitis is still unclear.

Gram-positive bacteria express a range of virulence factors. Cell-associated virulence factors like lipoteichoic acid (LTA) and lipoproteins are representative virulence factors that are recognized by Toll-like receptor 2 (TLR2) (Brightbill et al., 1999; Schwandner et al., 1999). These cell-associated virulence factors stimulate a variety of host cells and induce pro-inflammatory cytokines and chemokines (Hong et al., 2017; Kang et al., 2015). For instance, LTA of *Staphylococcus aureus* or *Streptococcus pyogenes* has been shown to induce IL-8 production in human peripheral blood monocytes (Standiford et al., 1994). Moreover, *S. aureus* lipoproteins were shown to induce IL-8 production in human intestinal epithelial cells (Kang et al., 2015). Likewise, LTA, lipoproteins, and peptidoglycan from *S. gordonii* induce production of pro-inflammatory cytokines, such as IL-6 and TNF, in dendritic cells via TLR2 (Mayer et al., 2009). However, little is known about the virulence factors of *S. gordonii* involved in the induction of inflammatory responses in the periapical areas. Therefore, in this study, we investigated the role of *S. gordonii* cell wall-associated virulence factors on IL-8 induction in human periodontal ligament (PDL) cells.

2. Materials and methods

2.1. Reagents

Todd-Hewitt broth was purchased from Kisan Bio (Seoul, Korea), and yeast extract was from BD Biosciences (San Diego, CA, USA). Triton X-114 and octyl β-D-glucopyranoside were purchased from Sigma-Aldrich (St. Louis, MO, USA). Alpha Modification of Eagle's Minimum Essential Medium (α-MEM), Dulbecco's modified Eagle's medium (DMEM), and penicillin-streptomycin solution were purchased from HyClone (Logan, UT, USA). Fetal bovine serum (FBS), trypsin-EDTA, and Opti-MEM were from Gibco-BRL (Carlsbad, CA, USA). Anti-human TLR2 antibody, its isotype control, and blasticidin S were purchased from InvivoGen (San Diego, CA, USA). TRIzol reagent was from Invitrogen (Grand Island, NY, USA). Random hexamers and reverse transcriptase were from Promega Corporation (Madison, WI, USA). Inhibitors for MAP kinases were purchased from Calbiochem (Darmstadt, Germany). EmeraldAmp GT PCR Master Mix was from TaKaRa (Otsu, Shiga, Japan).

2.2. Bacteria culture, bacterial inactivation, and cell culture

Wild-type CH1 *S. gordonii*, an *ltaS*-deficient mutant (Δ*ltaS*), and an *lgt*-deficient mutant (Δ*lgt*) were cultured to mid-log phase in Todd-Hewitt broth with 0.5% yeast extract (THY) at 37 °C (Kim et al., 2017). For preparation of ethanol-inactivated *S. gordonii*, bacteria were inactivated by 70% ethanol with shaking for 3 h. After washing with phosphate-buffered saline (PBS), no bacterial colonies were observed on THY agar plates after 2 days of growth (data not shown). The preparation and use of human PDL cells were as previously described (Im et al., 2015). The cells were grown in α-MEM containing 10% FBS and 1% penicillin/streptomycin at 37 °C in a 5% CO_2 incubator. Human embryonic kidney (HEK) 293 cells expressing TLR2 (HEK293-TLR2) were purchased from InvivoGen. The cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 10 μg/ml of blasticidin S at 37 °C in a 5% CO_2 incubator.

2.3. Cell transfection

HEK293-TLR2 cells were transfected as previously described (Lee et al., 2015). Briefly, HEK293-TLR2 cells (2.5×10^5 cells/ml, 5 ml, 60 mm dishes) were transfected with an NF-κB reporter gene construct (pNF-κB-Luc, Clontech, Mountain View, CA, USA). Transfections were performed in Opti-MEM using Attractene transfection reagent for 16 h (Qiagen, Germantown, MD, USA). After collection, the cells (2.5×10^5 cells/ml, 200 μl, 96-well plates) were plated in complete DMEM. The cells were then stimulated with *S. gordonii*, lipoprotein, LTA, or Pam2CSK4 for 16 h and lysed with GloLysis Buffer (Promega, Madison, WI, USA). The amount of luciferase activity in the cytoplasmic extracts was assayed using a luminometer (Molecular Devices, Sunnyvale, CA, USA).

2.4. Preparation of LTA from S. gordonii

Purified LTA was prepared from *S. gordonii* as previously described (Ryu et al., 2009). Briefly, bacteria were suspended in 0.1 M sodium citrate buffer (400 ml, pH 4.7), followed by ultrasonication. After mixing with an equal volume of *n*-butanol for 30 min, the reaction was centrifuged to separate the phases. The aqueous phase was dialyzed with a semi-permeable dialysis membrane (Spectra/Por 6; Spectrum® laboratories Inc., Ranch Dominquez, CA, USA) against endotoxin-free distilled water (Dai Han Pharm Co. Ltd., Seoul, Korea), after which it was solubilized in 15% n-propanol in 0.1 M sodium acetate buffer (pH 4.7). After hydrophobic-interaction chromatography with an Octyl-Sepharose column CL-4B (2.5 cm × 10 cm) (Sigma-Aldrich, MO, USA), unbound molecules were washed with 15% *n*-propanol in 0.1 M sodium acetate buffer (200 ml, pH 4.7). After elution of bound molecules using 35% *n*-propanol in 0.1 M sodium acetate buffer (300 ml), the inorganic phosphate assay was performed. Next, column fractions (including LTA) were dialyzed against endotoxin-free water, followed by DEAE-Sepharose ion-exchange chromatography (FastFlow, Sigma-Aldrich, 1.5 × 10 cm) in 0.1 M sodium acetate buffer (pH 4.7) including 30% n-propanol. After elution with 0.1 M NaCl in sodium acetate buffer (300 ml), the LTA was collected, pooled, and lyophilized. The quantity of purified LTA was determined through dry weight measurement.

2.5. Preparation of S. gordonii lipoproteins

Purified and putative lipoproteins were isolated from *S. gordonii* as previously described (Li et al., 2008). Briefly, bacterial pellets were collected and suspended in Tris-buffered saline (TBS) with protease inhibitors. After sonication, the bacterial lysates were suspended in a final concentration of 2% Triton X-114 at 4 °C for 2.5 h. Cell debris was discarded after centrifugation. The supernatant was incubated at 37 °C for 15 min and centrifuged at 37 °C to separate into an aqueous phase and a Triton X-114 phase. After discarding the aqueous phase, an equal volume of TBS was added to the Triton X-114 phase, after which the mixture was incubated at 37 °C for 15 min. After centrifugation, the Triton X-114 phase was incubated overnight with methanol at −20 °C. The precipitated lipoproteins were dissolved in 10 mM octyl-beta-D-glucopyranoside in PBS.

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

Human PDL cells were plated at a density of 3×10^5 cells/ml in a 6-well plate. After stimulation with various concentrations of