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Short communication

# Lipoproteins of *Actinomyces viscosus* induce inflammatory responses through TLR2 in human gingival epithelial cells and macrophages

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**Abstract**

*Actinomyces viscosus* has been suggested to be associated with periodontal disease. However, the pathogenicity of this bacterium is not known. In this study, we examined inflammation-inducing activity by *A. viscosus*. Whole cells and a lipophilic fraction of *A. viscosus* ATCC19246 induced production of interleukin-8 and tumor necrosis factor alpha from both human oral epithelial cells and human monocytoid cells. This cytokine production was blocked by lipoprotein lipase treatment of the lipophilic fraction. In addition, anti-Toll-like receptor 2 antibody blocked the cytokine production. These results suggest that lipoprotein of *A. viscosus* triggers inflammatory responses in periodontitis by activation of Toll-like receptor 2.


*Keywords: Actinomyces viscosus*; Inflammation; Lipoprotein; Toll-like receptor 2

## 1. Introduction

*Actinomyces viscosus* is a gram-positive oral bacterium that is frequently isolated from supragingival plaque and root surface caries lesions. Therefore, this bacterium has been suggested to be associated with periodontal inflammation. Although this bacterium has been implicated in the development of gingivitis [1–3], the pathogenicity of *A. viscosus* is poorly understood.

It is generally accepted that periodontal inflammation, including gingivitis, is caused by accumulation of dental plaque bacteria. Therefore, to better understand the mechanisms of this inflammation, it is important to identify the bacterial ligands and host cell receptors that contribute to the triggering of this response. Previous studies indicate that the proportion of *A. viscosus* increases significantly in the dental plaque at the site of gingivitis [2]. Additionally, disrupted water-soluble cell wall components of *A. viscosus* induce polyclonal B-cell activation in murine lymphocytes and activate murine macrophages to produce inflammatory cytokines [3–5]. These reports suggest that one or more *A. viscosus* cell wall molecules exhibit mitogenic, adjuvant, and inflammation-inducing activities. However, the cell wall components of *A. viscosus* that induce inflammation have not been identified, nor have the target molecules of human immune cells that serve as receptors for these bacterial components.

The host immune and epithelial cells produce Toll-like receptors (TLRs), type-1 transmembrane proteins that detect invasive microbes by recognition of various microbial components, collectively defined as pathogen-associated molecular patterns (PAMPs) [6]. Upon recognition of microbes through TLRs, these host cells release inflammatory cytokines. These cytokines are involved in initiation and amplification of the inflammatory responses in the host organisms. To date, more than 10 classes of TLRs have been identified. Among them, TLR2 has been reported to recognize the broadest range of PAMPs, including lipoprotein/lipopeptide, lipoteichoic acid, lipoarabinomannan, and yeast zymosan [7–11]. Interestingly, TLR2 also has been implicated

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in the inflammatory response triggered by several periodontopathic gram-negative bacteria [12,13], suggesting that TLR2 plays a key role in initiating periodontal inflammation. The molecular mechanisms of TLR2 binding have been demonstrated by X-ray crystallographic studies of TLR2 in the presence of synthetic lipopeptides [14–16]. However, the components of specific gram-positive bacteria that induce inflammatory responses through TLR2 are not fully understood. Recent reports have demonstrated that several well-known pathogenic gram-positive bacteria (specifically *Staphylococcus aureus*, *Listeria monocytogenes*, and *Streptococcus agalactiae*) activate host immune cells, and that TLR2 plays a key role in this response [17–20]. Additionally, these reports showed that bacterial lipoproteins from these species act as ligands of TLR2 to induce this response. In contrast to what is known in these bacteria, the inflammation-inducing mechanism(s) of gram-positive oral bacteria remain poorly understood. Furthermore, the predominant "sensors" that host cells use to detect gram-positive oral bacteria have not been identified. In the present study, we demonstrate that *A. viscosus* bacterial lipoproteins can induce periodontal inflammation, and are sensed by TLR2 present on periodontal cells of the host organism.

## 2. Materials and methods

### 2.1. Bacterial strain and culturing

*A. viscosus* ATCC19246 was obtained from the American Type Culture Collection (Manassas, VA). All bacterial culturing was performed at 37 °C under anaerobic conditions in brain heart infusion (BHI; Bacto™, NJ) agar or broth. Specifically, the strain was grown on plates for 3 days, then inoculated into broth and incubated overnight. The bacterial cells then were harvested by centrifugation (15,000 × *g*, 5 min, 4 °C), washed in phosphate-buffered saline (PBS), and resuspended at an optical density of 0.5 at 600 nm, corresponding to a cell density of approximately $1.1 \times 10^8$ colony-forming units (CFU)/ml.

### 2.2. Cell culture

Human embryonic kidney (HEK) 293 cells were obtained from the American Type Culture Collection (CRL-1573). These cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma–Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum, penicillin G (100 U/ml), and streptomycin (100 μg/ml). A human acute monocytic leukemia cell line, THP-1 (RCB1189), and a human cell line derived from oral squamous cell carcinoma, HSC-2 (RCB1145), were purchased from the Cell Engineering Division of RIKEN BioResource Center (Tsukuba, Ibaraki, Japan). Both lines were cultured in RPMI 1640 medium (Wako, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum. All cell culture was performed at 37 °C in a humidified 5% $CO_2$ atmosphere.

### 2.3. Antibodies and reagents

Mouse monoclonal antibody (mAb) to human TLR2 (TL2.1) and mouse IgG2a isotype control antibody were purchased from eBioscience (San Diego, CA). Phorbol 12-myristate 13-acetate, lipoprotein lipase from *Pseudomonas* sp., and Triton X-114 (TX-114) were purchased from Sigma–Aldrich.

### 2.4. Gene cloning

The cDNA of human *TLR2* was prepared by reverse transcription–polymerase chain reaction of RNA isolated from THP-1 cells and cloned into pEF6/V5-His-Topo vector (Invitrogen Co., Carlsbad, CA) using standard molecular biology techniques [21]. The resulting plasmid was designated pEF6-TLR2.

### 2.5. Extraction of lipophilic fraction of A. viscosus

*A. viscosus* cells were treated with TX-114 to extract the lipophilic fraction. Specifically, *A. viscosus* was cultured in BHI broth and harvested as described above. The cell pellet was resuspended in buffer solution (150 mM NaCl and 10 mM Tris–HCl, pH 8.0). The cell suspensions were mixed with 1/10 volume of 20% (vol/vol) aqueous TX-114 working stock solution. The mixture was rotated at 4 °C for 2 h and cell debris was removed by centrifugation (15,000 × *g*, 5 min, 4 °C). The supernatant was incubated for 5 min at 37 °C and re-centrifuged to separate the lower (lipophilic) phase from the upper (aqueous) phase. Excess methanol was added to the lower phase to precipitate the lipophilic fraction; the mixture was incubated overnight at −80 °C prior to centrifugation (15,000 × *g*, 30 min, 4 °C) and decanting/discarding of the supernatant. The precipitated lipophilic fraction was resuspended in PBS, and is hereafter referred to as Lipo-fract. The protein concentration of Lipo-fract was measured by BCA assay using BIO-RAD PROTEIN ASSAY (Bio-Rad Laboratories, Hercules, CA). To determine the stimulatory activity of lipoproteins contained in Lipo-fract, aliquots of Lipo-fract were incubated (37 °C, 6 h) in the presence or absence of lipoprotein lipase (98,100 U/ml) prior to use in stimulation.

### 2.6. ELISA

HSC-2 cells were plated at $1.0 \times 10^4$ cells per well in 24-well plates and cultured for 4 days (to achieve confluency). THP-1 cells were plated at $4.0 \times 10^5$ cells per well in 24-well plates in the presence of 10 nM phorbol 12-myristate 13-acetate and cultured for 24 h (to differentiate into macrophages). These cells were washed three times with serum-free RPMI 1640 and then were stimulated for 6 h with *A. viscosus* cells ($1.1 \times 10^5$, $1.1 \times 10^6$, and $1.1 \times 10^7$ CFU/ml) or Lipo-fract (at protein concentration of 0.01, 0.1, and 1 μg/ml) in serum-free medium. After stimulation, the culture supernatants were collected by centrifugation (7000 × *g*, 10 min, 4 °C). The amounts of interleukin-8 (IL-8) and tumor necrosis factor alpha (TNF-α) in the culture supernatants were determined using enzyme-linked