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ORIGINAL ARTICLE

Involvement of Toll-like receptor 2 in apoptosis of *Aggregatibacter actinomycetemcomitans*-infected THP-1 cells

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KEYWORDS
Aggregatibacter (*Actinobacillus*) *actinomycetemcomitans*;
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Background/Purpose: Aggregatibacter (*Actinobacillus*) *actinomycetemcomitans* is a gram-negative bacterium that has been associated with aggressive periodontitis. *A actinomycetemcomitans* infection induces apoptosis in the human monocytic cell line THP-1, and p38 mitogen-activated protein kinase (p38) activity and tumor necrosis factor-α (TNF-α) production are enhanced by *A actinomycetemcomitans* infection. However, mechanisms governing the recognition of *A actinomycetemcomitans* by monocytes during apoptosis have not been elucidated. *A actinomycetemcomitans* cell wall components stimulate Toll-like receptor (TLR)2 and/or TLR4. The authors examined whether TLR2 and/or TLR4 were involved in the apoptosis of *A actinomycetemcomitans*-infected THP-1 cells.
Methods: A actinomycetemcomitans-infected THP-1 cells were transferred to six-well culture plates and incubated for 0 to 6 hours. In some experiments, THP-1 cells were incubated with anti-TLR2, anti-TLR4, or isotype control antibody (10 μg/mL) for 30 minutes prior to *A actinomycetemcomitans* infection. Expression of messenger RNA (mRNA) was examined by reverse transcription-polymerase chain reaction. Intracellular bacteria recovered from *A actinomycetemcomitans*-infected cells and apoptotic cells were detected by APOPercentage dye (Biocolor Ltd, Northern Ireland, UK) staining. Cellular p38 activity and TNF-α production were assessed with enzyme-linked immunosorbent assay.

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Results: The expression of TLR2 mRNA was increased by *A actinomycetemcomitans* infection. Phagocytosis and apoptosis in *A actinomycetemcomitans*-infected THP-1 cells were inhibited by the addition of anti-TLR2 antibody. Also, anti-TLR2 antibody suppressed the activation of p38 and production of TNF-α in *A actinomycetemcomitans*-infected THP-1 cells.
Conclusion: These results suggest that *A actinomycetemcomitans* induces increased expression of TLR2, leading to phagocytosis and apoptosis of THP-1 cells via p38 activation and TNF-α production.

Introduction

Aggregatibacter (*Actinobacillus*) *actinomycetemcomitans* has been implicated in the pathogenesis of aggressive periodontal disease.[1] *A actinomycetemcomitans* possesses various virulence factors; in addition, *A actinomycetemcomitans* can invade periodontal tissue and survive inside host cells.[2] An earlier study developed an *in vitro* infection model for *A actinomycetemcomitans* to provide evidence for the apoptosis of human epithelial cell line KB and human monocytic cell line THP-1 after infection. The invasion of *A actinomycetemcomitans* into KB cells induced apoptosis.[3,4] This mechanism could occur in gingival epithelial cells, inducing apoptosis without inflammation. We have shown that the induction of apoptosis in *A actinomycetemcomitans*-infected THP-1 cells occurs via the p38 mitogen-activated protein kinase (p38) pathway.[4] The mechanisms governing the recognition of *A actinomycetemcomitans* by monocytes during apoptosis have not yet been elucidated.

Various pattern recognition receptors (PRRs) in innate immune cells recognize specific structures of microorganisms. The target of innate immune recognition is the conserved bacterial pathogen-associated molecular patterns (PAMPs) of microorganisms including lipopolysaccharide (LPS), lipoprotein, peptidoglycans, and lipoteichoic acid.[5] PAMPs are essential for microbial survival and are conserved structures among many pathogens, allowing innate immunity to recognize microorganisms with remitted numbers of PRRs. Among PRRs, Toll-like receptors (TLRs) have been highlighted as key recognition homologues of the innate immune system.[6]

TLRs belong to a family of leucine-rich repeat proteins that are either expressed at the cell surface or in the intracellular compartments of inflammatory cells.[7] Twelve types of TLRs have been identified in humans, each of which recognizes a particular ligand.[6,8] TLR2 and TLR4 are the principal signaling receptors for bacterial cell wall components. In general, LPS of gram-negative bacteria is recognized by TLR4. TLR2 recognizes various bacterial components, including peptidoglycans, lipopeptide, and lipoprotein. [6,7,9,10]

In periodontal tissues, TLR2 and TLR4 are involved in the progression of inflammation and are protective against bacterial infection. [11,12] Increased expression of TLR2 and TLR4 was observed in inflamed gingival tissues.[11] Although the cell wall components of most gram-negative periodontal bacteria exclusively stimulated TLR2, *A actinomycetemcomitans* was capable of stimulating both TLR2 and TLR4.[12] Both TLR2 and TLR4 are expressed on antigen-presenting cells such as macrophages and dendritic cells.[13,14] These TLRs stimulate activation of the cells and the production of cytokines including interleukin (IL)-1β and tumor necrosis factor (TNF)-α, resulting in an adaptive immune response.[15] TLR signaling often induces apoptosis in certain types of cells.[16,17]

We hypothesized that apoptosis in *A actinomycetemcomitans*-infected THP-1 cells is induced by the activation of TLR2 and/or TLR4.

Methods

Cells and growth conditions

A actinomycetemcomitans Y4 strain was grown in Todd-Hewitt broth (Becton Dickinson, Cockeysville, MD, USA) supplemented with 1% yeast extract at 37°C for 1 day in an atmosphere of 5% CO_2 in air. Human monocytic THP-1 cells (JCRB0112.1; JCRB, Tokyo, Japan) were maintained in RPMI 1640 medium (Sigma-Aldrich Chemical Company, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich), penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37°C in 5% CO_2 in air.

In vitro infection procedure

THP-1 cells were infected with *A actinomycetemcomitans* according to the procedures described in our previous study. Briefly, THP-1 cells were suspended in microtubes at a concentration of 2×10^7 cells/mL. *A actinomycetemcomitans*, which was harvested by centrifugation and suspended in RPMI 1640 medium without antibiotics, was added to THP-1 cells in microtubes at a bacterium-to-cell ratio of 1000:1; subsequently, tubes were centrifuged at 1000 $\times$ g for 10 minutes at 4°C. Next, the tubes were incubated in RPMI 1640 medium containing 5% FBS without antibiotics at 37°C for 30 minutes. Infected cells were washed via a series of centrifugations with RPMI 1640 medium supplemented with penicillin, streptomycin, and gentamicin (200 μg/mL) to remove nonadherent bacteria. After transfer to six-well culture plates, the infected cells were incubated in RPMI 1640 medium supplemented with 5% FBS, penicillin, streptomycin, and gentamicin for 0 to 6 hours. Anti-TLR2, anti-TLR4 (Imgenex, San Diego, CA, USA; 10 μg/mL), or isotype control antibody (Sigma-Aldrich;