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Effect of Various Agents on Oral Bacterial Phagocytosis in THP-1 Cells

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Phagocytosis is a fundamental process in which phagocytes capture and ingest foreign particles including pathogenic bacteria. Several oral pathogens have anti-phagocytic strategies, which allow them to escape from and survive in phagocytes. Impaired bacteria phagocytosis increases inflammation and contributes to inflammatory diseases. The purpose of this study is to investigate the influences of various agents on oral pathogenic phagocytosis. To determine phagocytosis, Streptococcus mutans, Fusobacterium nucleatum, Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis were stained with 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE), and was measured using flowcytometery and confocal microscopy. The influencing factors on phagocytosis were evaluated through the pretreatment of ROS inhibitor (N-acetyl-L-cysteine (NAC)), lysozyme, potassium chloride (KCI) and adenosine triphosphate (ATP) in THP-1 cells. Expression of pro-inflammatory cytokines was determined by enzyme-linked immunosorbent assay (ELISA). The phagocytosis of various bacteria increased in a MOI-dependent manner. Among the tested bacteria, phagocytosis of P. gingivalis showed the highest fluorescent intensity at same infection time. Among the tested inhibitors, the NAC treatment significantly inhibited phagocytosis in all tested bacteria. In addition, NAC treatment indicated a similar pattern under the confocal microscopy. Moreover, NAC treatment significantly increased the bacteria-induced secretion of IL-1β among the tested inhibitors. Taken together, we conclude that the phagocytosis occurs differently depending on each bacterium. Down-regulation by ROS production inhibited phagocytosis and lead increased of oral pathogens-associated inflammation.

Key words: ***Streptococcus mutans (S. mutans), Fusobacterium nucleatum (F. nucleatum), Aggregatibacter actinomycetemcomitans (A. actinomycetemcomitans), Porphyromonas gingivalis (P. gingivalis),*** **phagocyotosis, macrophage, monocyte**

Introduction

Phagocytosis is a complex process for the ingestion and elimination of pathogens [1]. It is also important for elimination of apoptotic cells and for maintaining tissue homeostasis [2]. Phagocytosis is the hallmark of specialized cells including monocytes, macrophages, dendritic cells, osteoclasts and neutrophils. These cells are collectively referred to as professional phagocytes [3]. The phagocytic process is very efficient and ends in the destruction of invaded micro-organisms. The presence of regulators that affect phagocytosis may alter the phagocytic capacity of professional phagocytes [4]. Phagocytosis of cariogenic streptococci has been reported to be enhanced by specific antibody [5]. Probiotics can activate phagocytosis of

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macrophages against *Aggregatibacter actinomycetemcomitans* [6]. Thus, understanding the regulatory mechanism on phagocytosis process can be the important strategies against bacteria infection.

Phagocytosis affects a series of inflammatory response. Inflammatory response through phagocytosis constitute the first line of defense which induce the inflammatory cytokine production and cellular recruitment [7]. The removal of dying cells or invaded pathogens by phagocytes occurs without eliciting an inflammatory response and is an important function of phagocytosis [8]. Chronic periodontitis is also associated with impaired phagocytosis [9]. A significant reduction in phagocyte functions was observed in individuals with periodontitis [10]. However, the influencing factors of phagocytosis on various oral pathogens in monocyte remain to be elucidated. The purpose of this study was to investigate the influences of various inhibitors on oral pathogen phagocytosis. Furthermore, we investigated whether the regulation of phagocytosis influenced inflammatory response in THP-1 cells.

Materials and Methods

Bacterial culture

Streptococcus mutans (S. mutans) was grown in brain-heart infusion (BHI) broth (BD, Franklin Lakes, USA) broth at 37˚C in a 5% CO_2 incubator. *Porphyromonas gingivalis (P. gingivalis)* and *Fusobacterium nucleatum (F. nucleatum)* were grown in Gifu anaerobic medium (GAM; Nissui, Japan) broth, which contained hemin (5 mg/mL) and 3-phytyl-menadione (vitamin K, 0.5 mg/ml) at 37°C in an anaerobic chamber under an atmosphere containing 90% N_2, 5% H_2, and 5% CO_2. *Aggregatibacter actinomycetemcomitans (A. ctinomycetemcomitans)* was grown in tryptic soy (TBS) broth (BD, Franklin Lakes, USA) with 1% yeast extract (LPS solution, Seoul, South Korea) at 37˚C in a 5% CO_2 incubator. Each bacterial culture was harvested by centrifugation at 5000 rpm for 5 min, resuspended in RPMI media (Gibco, CA, USA) and used to infect the macrophages at a multiplicity of infection (MOI).

Cell culture and treatment

The human monocyte cell line, THP-1 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and were cultured at 37°C in 5% CO_2 incubator. For phagocytosis assay, THP-1cells were infected with CFSE-stained bacteria for 45 min with or without the pretreatment of N-Acetyl-L-cysteine (NAC, sigma, MO, USA), Potassium chloride (KCI, sigma, MO, USA), Adenosine 5′-triphosphate disodium salt hydrate (ATP, sigma, MO, USA) and lysozyme (sigma, MO, USA) for 30 min. For confocal microscopic examination, THP-1 cells were differentiated into macrophage-like cells with 50 ng/ml of Phorbol12-mystristate13-acetate (PMA; sigma, MO, USA).

Phagocytosis assay

THP-1 cells were seeded at 4.0×10^5cells/well in a 24 well plate. Bacteria were labeled with 10μM Cell race™ CFSE cell proliferation kit (Thermo Fisher Scientific, MA, USA) in PBS containing 0.1% BSA for 15 min. THP-1cells were infected CFSE stained-bacteria for 45 min. After infection, cells were washed with cold PBS and analyzed using a FACSVerse flowcytometer (Becton Dickinson, CA, USA). For confocal microscopic examination, THP-1 cells were seeded in 8-well-chamber slides. Cells were pre-treated with or without NAC (5 mM), KCI (50 mM), ATP (20 μM) and lysozyme (50 μg/ml) for 30min followed by infection with bacteria (MOI 50) for 24h and stained with 4',6-Diamidino-2-Phenylindole (DAPI, Thermo Fisher Scientific, MA, USA). Then, cells were washed with PBS and fluorescent micrographs were obtained using confocal laser-scanning microscope (LSM700, CarlZeiss, Oberkochen, Germany).

Cytokine production analysis

To determine the concentration of cytokines released into the culture media after bacterial infection, the supernatant was analyzed using an ELISA kit (Biolegend, CA, USA) according to manufacturer's instructions. The plates were read using an ELISA reader (Tecan, Männedorf, Switzerland) at 450/570 nm.

Statistics

Statistically significant differences between samples were analyzed with the SPSS 21.0 statistical software program (SPSS Inc., IL, USA). The data were shown as the mean ± SD. A p value of < 0.05 was considered statically significant.

Results

Phagocytosis of various oral bacteria increased in a MOI-dependent manner

To determine if bacteria induce phagocytosis in THP-1 cells, phagocytosis was examined by CFSE-stained bacterial infection

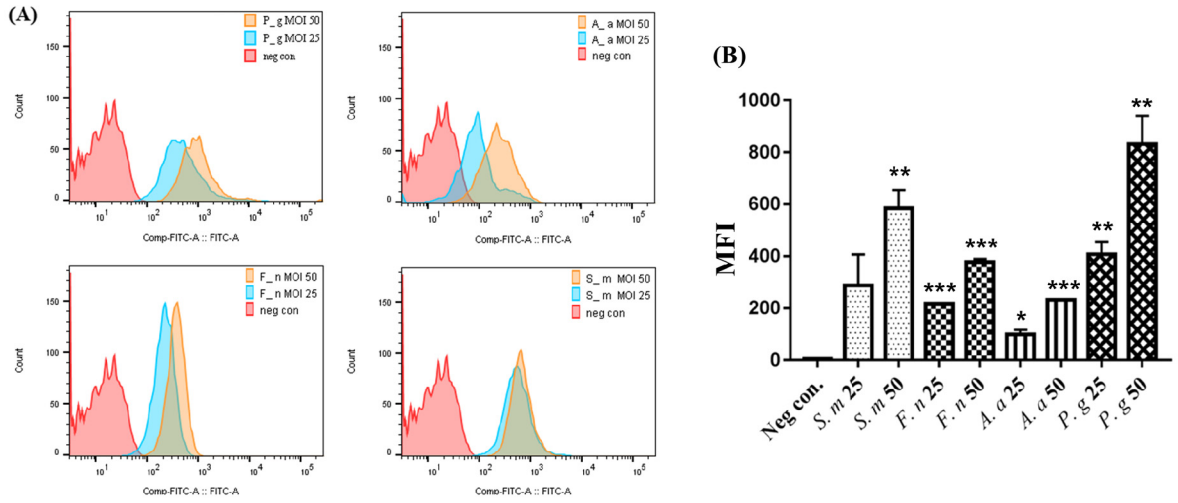

Fig 1. Phagocytosis of various bacteria were shown in a MOI-dependent manner.
(A) THP-1 cells were infected with *S. mutans (S. m), P. gingivalis (P. g), F. nucleatum (F. n)* and *A. actinomycetemcomitans (A. a),* respectively (MOI 25 and 50) for 45 min. The phagocytic uptake was assessed with flowcytometric analysis. (B) Phagocytic intensity of each group was shown in bar graphs. Mean Fluorescent Intensity (MFI) *p<0.05, ** p<0.01, *** p<0.001

and was quantitated by flow cytometry. THP-1 cells infected with oral bacteria showed significantly increased mean fluorescence intensity (MFI) in a MOI-dependent manner (Fig. 1). Among the bacteria, phagocytosis for *P. gingivalis* showed the highest MFI in THP-1 cells at same infection time.

Treatment of ROS inhibitor N-acetyl-L-cysteine (NAC) significantly inhibited phagocytosis of the THP-1 cells

To determine the influencing factor of phagocytosis by bacterial infection, we pretreated NAC, KCl, ATP and lysozyme and the degree of phagocytosis was analyzed by flow cytometry. NAC pretreatment suppressed the phagocytosis of all tested oral bacteria infected THP-1 cells significantly (Fig. 2A).

To confirm the effect of NAC on phagocytosis against oral bacteria, THP-1 cells were infected with the CFSE-stained bacteria and were stained with DAPI. Among the CFSE-labeled bacteria, *F. nucleatum*uptake was representatively examined with confocal microscopy. After the infection, CFSE-labeled *F. nucleatum* was found in THP-1 cells and NAC pretreatment markedly reduced the intracellular bacteria suggesting that NAC inhibited the phagocytosis of *F. nucleatum* in THP-1 cells (Fig. 2B).

NAC treatment significantly increased the bacteria-induced secretion of inflammatory cytokine IL-1β

Finally, we tested the relation between phagocytosis and inflammatory response in the bacteria-infected THP-1 cells. Each bacterial infection induced IL-1β production in THP-1 cells significantly and NAC pretreatment increased IL-1β secretionc ompared to bacterial infection alone significantly. These results indicate that ROS inhibition significantly increased inflammatory cytokine production in THP-1 cells.

Discussion

Periodontitis is an inflammatory disease caused by periodonto-pathogens and functional phagocytes to clear pathogens is important for periodontal health (11). The phagocytic process is usually very efficient and leads to the destruction of ingested micro-organisms (12). However, relation between phagocytosis of various oral pathogen and inflammatory response is not clear. Thus, in this study, we compared phagocytosis among oral bacteria including periodonto-pathogens and *S. mutans*, and further examined the effect of influencing factors on phagocytosis and inflammatory cytokine production.

Phagocytosis is essential innate immune defense mechanism for clearing bacterial infection (13). In this study, to examine if there is any difference of phagocytosis among oral bacteria, we infected the bacteria to THP-1 cells and examined the degree of phagocytosis by flow cytometry. Phagocytosis was increased in THP-1 cells infected with all the tested bacteria in a MOI-dependent manner. Phagocytosis initiates with the recognition and ingestion of microbial pathogens into a phagosome. This

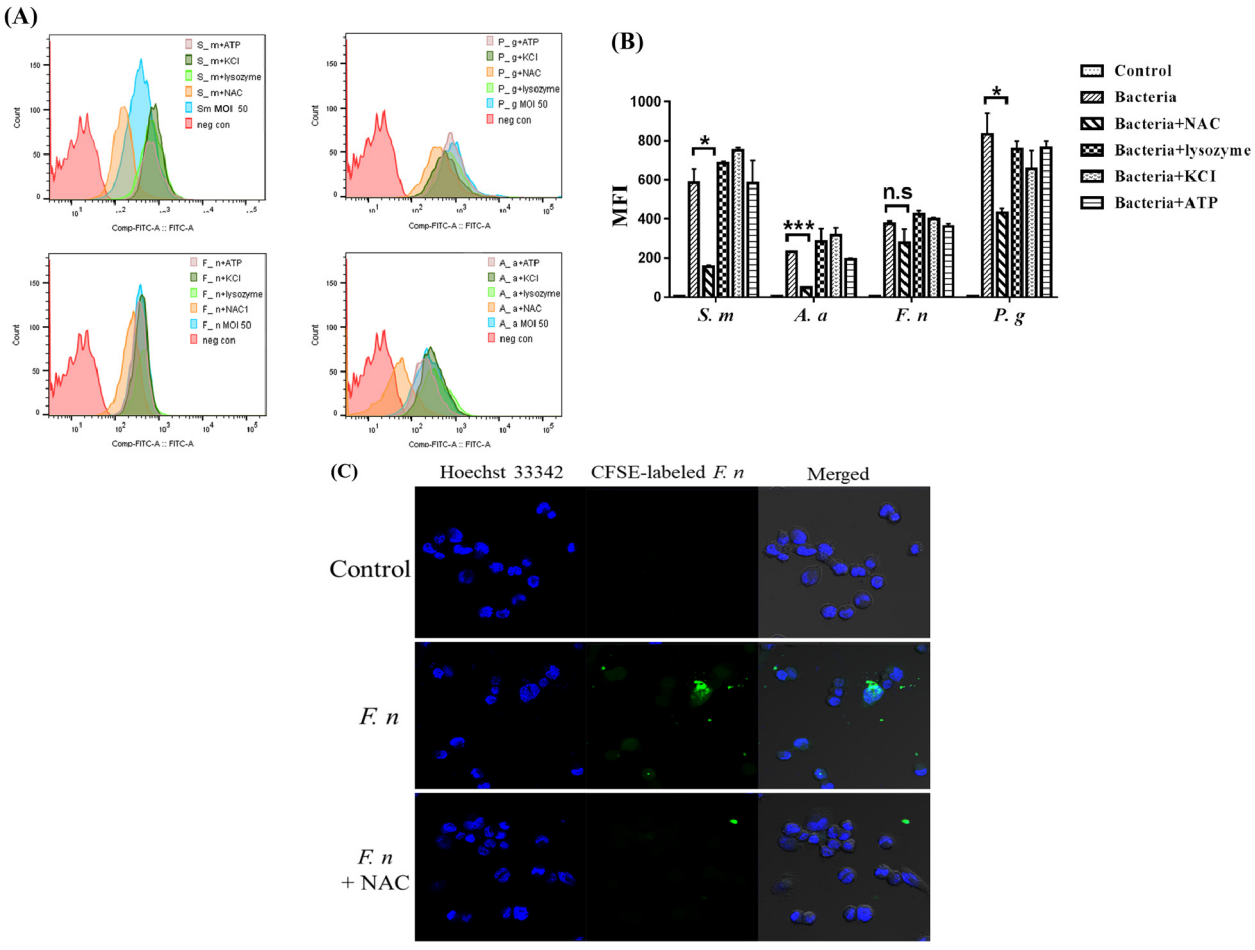

Fig 2. Treatment of ROS inhibitor N-acetyl-L-cysteine (NAC) significantly inhibited phagocytosis of the THP-1 cells.
THP-1 cells were pretreated with of ROS inhibitor NAC (5 mM), KCI (50 mM), ATP (20 μM) and lysozyme (50 μg/ml) respectively and then infected with the bacteria for 45 min. (A) The phagocytic uptake was assessed with flowcytometric analysis. (B) Phagocytic intensity of each group was shown in bar graphs. (C) The CFSE-labeled *F. nucleatum (F. n)* uptake was representatively visualized with confocal microscopy. The nucleus was stained with DAPI (blue). 400X. *p<0.05, ** p<0.01, *** p<0.001.

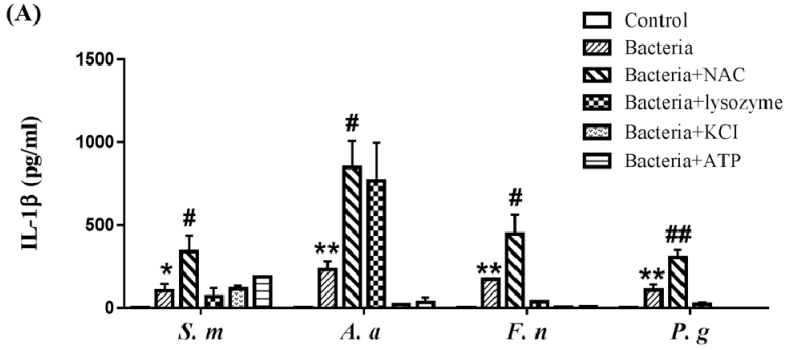

Fig 3. The NAC treatment significantly increased the bacteria-induced secretion of inflammatory cytokine IL-1β.
(A) THP-1 cells were pretreated with of ROS inhibitor NAC (5 mM), KCI (50 mM), ATP (20 μM) and lysozyme (50 μg/ml) respectively and then infected with bacteria for 24 hours. The culture supernatant was analyzed for the determination of IL-1β production by ELISA. *p<0.05, ** p<0.01 versus control. # P <0.05 ## p<0.01 versus bacterial infection.

recognition is achieved through receptors that recognize molecular patterns associated with the bacteria (14). Binding of phagocytic receptors leads to localized actin polymerization at the site of ingestion (15). Several downstream signaling

cascades occur during phagocytosis, including protein tyrosine kinases (16), phosphatidylinositol 3- kinase (17) and protein kinase C (18). Thus, initial phagocytosis in tightly regulated by the recognition and subsequent signaling cascades.

To examine the effect of chemical inhibitors for controlling phagocytosis, we pretreated several reagents that are found in saliva or is related with ROS production. Among the tested inhibitors, NAC pretreatment significantly suppressed phagocytosis, indicating ROS production is associated with phagocytosis process. In microbial killing step of phagocytosis, it was classified with oxygen-independent means or oxygen-dependent means called the 'respiratory burst' (19). Oxygen-independent process includes a changed acid environment and release of defensins, hydrolytic enzymes, and lactoferrin from lysosomes. Oxygen-dependent process reported that the phagolysosome generates enzymes leading to the release of reactive forms of oxygen (20). Production of ROS is achieved by the NADPH oxidase (NOX2) on the membrane of phagosome (21). The reactive nitrogen intermediates (nitric oxide (NO), nitrite(NO_2-), nitrate(NO_3-) are also produced to destroy micro-organisms in phagocytes (22). Thus, inhibition of ROS production by NAC may have blocked the phagocytosis by influencing the survival of bacteria against ROS products (Fig2A). We have confirmed these results in *F. nucleatum*-infected THP-1 cells using confocal microscopy, because the phagocytosis of *F. nucleatum* was the least affectedby NAC pretreatment. CFSE-labeled *F. nucleatum*was detected in THP-1 cells and NAC pretreatment markedly decreased the intracellular CFSE fluroescent intensities suggesting that NAC pretreatment inhibited the internalization of *F. nucleatum* (Fig 2B).

Many studies demonstrated that the role of phagocytosis in inflammatory response. As inflammatory barriers, influx of phagocytic cells into infected area occurs (23). The neutrophils which arrive on the lesion first phagocytize invading bacteria and release chemical mediators (24). Subsequently, macrophage become activated to phagocytic bacteria and release the inflammatory cytokines such as interleukin 1, interleukin 6 and tumor necrosis factor (TNF)-α. In periodontitis, phagocytosis by phagocytes constitutes the initial defense mechanism which have the functional consequence against bacterial challenges (26).

The primary function of cytokines is to regulate infl ammation, and as such, play a vital role in regulating the immune response in health and disease (27). Inflammatory cytokine, IL-1β binds to a specific cell surface receptor to cascade a cellular proliferation, metabolism, chemotaxis, and tissue repair. However, excessive inflammatory responses frequently cause problems that lead to oral diseases including gingivitis and periodontitis (28). In this study, we showed that phagocytosis inhibition by NAC treatment led to the increased production of IL-1β. These data suggest that decreased phagocytic function by ROS blocking caused excessive production of IL-1β may continually aggravate the inflammation in THP-1 cells.

In summary, the phagocytosis efficiency differs depending on each bacterium and inhibition of ROS production by NAC suppressed phagocytosis and led to increased inflammatory cytokine IL-1β production. Thus, the regulation of ROS expression may provide a new strategy for the control of periodontal inflammation via modulating phagocytosis.

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Conflict of interest

The author's declare that there is no conflict of interest that would prejudice the impartiality of this work.

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