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Original Article

Effect of *Weissella cibaria* on *Fusobacterium nucleatum*-induced Interleukin-6 and Interleukin-8 Production in KB Cells

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Oral microorganisms, including pathogens together with commensals, interact with oral epithelial cells, which can lead to the activation and expression of a variety of inflammatory mediators in epithelial cells. *Fusobacterium nucleatum* is a filamentous human pathogen that is strongly associated with periodontal diseases. Our previous data suggest that *Weissella cibaria*, an oral commensal, inhibits the proliferation of periodontopathic bacteria including *F. nucleatum*. The aim of this study was to examine the effects of *W. cibaria* on the inflammatory mediators, interleukin (IL)-6 and IL-8, in KB cells stimulated by *F. nucleatum*. In a reverse transcription-polymerase chain reaction and an enzyme-linked immunosorbent assay, live *F. nucleatum* alone induced high levels of gene expression and protein release of IL-6 and IL-8, whereas *W. cibaria* alone did not induce IL-6 and IL-8 responses in KB cells. *W. cibaria* dose-dependently inhibited the increases of the IL-6 and IL-8 gene expression as well as IL-6 protein level in KB cells which was induced by *F. nucleatum*. Bacterial viability and its coaggregation with *F. nucleatum* are not essential in the inhibitory effect of *W. cibaria*. Visible effects of *W. cibaria* on the attachment and invasion of KB cells by *F. nucleatum* were observed. In conclusion, *W. cibaria* may exert immunomodulatory effects on the IL-6 and IL-8 responses to *F. nucleatum*-activated KB cells.

Key Words: *Weissella cibaria*, *Fusobacterium nucleatum*, Interleukin-6, Interleukin-8, KB cells

INTRODUCTION

Periodontitis, an inflammatory disease of the tooth supporting tissues, is initiated by an overgrowth of specific gram-negative anaerobic bacteria that leads to gingival connective tissue destruction and irreversible alveolar bone resorption. It has been shown that a group of microbes, such as *Porphyromonas gingivalis*, *Tannerella forsythia*, *Aggregatibacter actinomycetemcomitans*, *Treponema denticola*, and *Fusobacterium nucleatum*, exist in complexes in subgingival plaque (1~3), and they are suspected to be the putative agents of periodontal diseases. Among these bacteria, *F. nucleatum* is not only predominant in terms of its numbers in the oral cavity, but also possesses the capacity to form aggregates with other bacteria, and to function as a bridge between the primary and secondary settlers on the surfaces of the teeth (4). In addition, this microorganism is a potentially important regulator of the host response due to its ability to invade cells and tissues and subsequently modify the immune response (5).

Epithelial cells act as a physical barrier and function as a sensor for the presence of bacteria. Direct physical contact between bacteria and the mucosal surface triggers the expression of a variety of immune response mediators from

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epithelial cells (6, 7). The continuous high level secretion of various cytokines such as interleukin (IL)-6 and IL-8 by host cells following stimulation with periodontopathogens and their products is a critical determinant of periodontal tissue destruction (7, 8). IL-6, a multifunctional cytokine, plays an important role in regulating the immune response during periodontal disease (9). IL-6 expression was found to be higher at sites of periodontal inflammation and closely related to the clinical severity of periodontitis (10). IL-8 is a pro-inflammatory chemokine that attracts and activates neutrophils. IL-8 is up-regulated in oral or gingival epithelial cells after a challenge with several periodontal bacteria (5, 11, 12). The regulation of inflammatory reactions in host cells has been suggested to be one way of controlling the progression of periodontitis (13).

Oral microorganisms, including pathogens together with commensals, interact with oral epithelial cells, which can lead to the activation and expression of a variety of inflammatory mediators in epithelial cells. These observations raise questions regarding how intracellular microbial infections occur and how host cells respond under those conditions. Emerging evidence has shown that commensal bacteria behave in a manner different from that exhibited by pathogenic bacteria, in that they induce very low levels of pro-inflammatory cytokines (14). Some commensal gut bacteria have been shown to be capable of attenuating the acute inflammatory cytokine responses triggered in intestinal epithelial cells (15, 16). These findings suggest that commensal bacteria might contribute to immunological homeostasis at epithelial surfaces. Whether similar mechanisms take place in the oral environment has not been elucidated.

Our study has approached these questions by employing *F. nucleatum* and *Weissella cibaria* as model organisms for the simultaneous infection of epithelial cells. *W. cibaria*, an oral commensal, is a lactic acid bacterial (LAB) species, and was formerly included in the genus *Lactobacillus* (17). Recently, we previously suggested that *W. cibaria* isolates from human saliva may be used as a probiotic due to its inhibitory effect on biofilm formation and volatile sulfur compound formation as well as the proliferation of five periodontopathic bacteria including *F. nucleatum* (18~20). The aim of the present study was to examine the effects of *W. cibaria* on the production of inflammatory mediators, IL-6 and IL-8, by KB oral epithelial cells stimulated with *F. nucleatum*.

MATERIALS AND METHODS

Bacterial strains and culture conditions

W. cibaria CMU was isolated from the saliva of healthy children, between 4 and 7 years of age, all of whom lived in Gwangju, South Korea. *W. cibaria* was grown in De Man, Rogosa, Sharpe broth (MRS broth; Difco, Detroit, MI, USA) at 37℃ for 16 h under aerobic conditions. For the preparation of killed *W. cibaria*, the bacteria were harvested, washed three times with phosphate-buffered saline (PBS), and resuspended in a small volume of PBS. The bacteria were then exposed to heat (100℃ for 10 min) or formalin (4% formalin for 20 min). The formalin-treated bacteria were washed extensively with PBS, and resuspended again in a small volume of PBS. *F. nucleatum* ATCC 10953 was grown in trypticase soy broth (Difco) supplemented with yeast extract (1 mg/ml), hemin (10 μg/ml; Sigma, St Louis, MO, USA), and menadione (5 μg/ml; Sigma) under anaerobic conditions (85% N_2, 10% H_2, 5% CO_2) at 37℃ for 48 h (21). To determine the effect of direct contact between *W. cibaria* and host cell, *W. cibaria* was separated from KB cells and *F. nucleatum* through a tissue culture insert (pore size 0.45 μm; Corning Inc., Corning, NY, USA) in the co-culture system for 24 h. For inhibition of bacterial coaggregation, *F. nucleatum* was pre-incubated with 1 mM L-canavanine (Sigma), an analogue of L-arginine that inhibits coaggregation, for 15 min (22). For comparison of other lactic acid bacteria (LAB), *Lactobacillus acidophilus* ATCC 4356, *L. casei* KCTC 3260, *L. fermentum* ATCC 43931 and *L. reuteri* KCTC 3594 were used in this study. These bacteria were also grown in MRS broth at 37℃ for 16 h under aerobic conditions. Before being used in each experiment, they were propagated twice in an appropriate broth.

Cell culture and bacterial infection

KB cells (a human mouth epithelial cell line, KCLB 10017) were grown in 5% CO_2 at 37℃ in minimal essential medium containing Earle's salts (Hyclone, Logan, Utah, USA), 2 mM L-glutamine and 10% fetal bovine serum. Bacterial cultures were harvested, washed twice with PBS and then resuspended in serum-free MEM. The optical density of the bacterial suspension was measured at 600 nm, and then diluted to an optical density of 0.5, which corresponded to 5×10^8 CFU/ml. Bacteria were added to cell monolayers at multiplicity of infection (MOI) of 1:100 and incubated for various periods of time at 37℃ in 5% CO_2. For the measurement of mRNA level, 6-well plates were seeded with 3×10^5 KB cells and incubated with *W. cibaria* and *F. nucleatum* for 4 h. For the cytokine assay, 24-well plates were seeded with 1×10^5 KB cells and incubated with *W. cibaria* and *F. nucleatum*. After incubation for 24 h, the supernatants were collected and stored at -20℃.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the KB cells using the Easy-BLUE™ RNA extraction kit (iNtRON Biotech, Sungnam, South Korea) according to the manufacturer's instructions. RT-PCR was performed using the ONE-STEP RT-PCR premix kit (iNtRON) as follows. Total RNA (500 ng) was reverse-transcribed to cDNA that was then amplified by PCR using oligonucleotide primers specific for IL-6, IL-8 or β-actin. The primer sequences were as follows: IL-6 (5'-TCAATGAGGAGACTTGCCTG-3'; 5'-GATGAGTTGTCATGTCCTGC-3') (23), IL-8 (5'-TGTGCTCTCCAAATTTTTTTTACTG-3'; 5'-CTCTCTTTCCTCTTTAATGTCCAGC-3') and β-actin (5'-AGCGGGAAATCGTGCGTG-3'; 5'-CAGGGTACATGGTGGTGCC-3') (24). β-actin was used to confirm equal amount of RNA loading for reverse transcription and PCR amplification under different experimental conditions. The amplified fragment sizes for IL-6, IL-8 and β-actin were 260 bp, 408 bp and 300 bp, respectively. The PCR products (10 μl) were fractionated on 1.5% agarose gels containing ethidium bromide (0.5 μg/μl), visualized by UV transillumination, and photographed. The sizes of the amplified DNA fragments were identified by comparison with a molecular size marker DNA (Invitrogen, Carlsbad, CA, USA).

Enzyme-linked immunosorbent assay (ELISA)

The levels of IL-6 and IL-8 in culture supernatants were quantified by ELISA kit (BioLegend, San Diego, CA, USA) according to the manufacturer's directions. Briefly, human IL-6 or IL-8 specific monoclonal antibody (Ab) was coated on 96-well microplates. Standards and samples were added to the wells. Subsequently, biotinylated anti-human IL-6 or IL-8 detection Ab was added, followed by avidin-horseradish peroxidase. TMB (3,3',5,5'-tetramethyl benzidine) substrate solution was added, producing a blue color in proportion to the amount of cytokines present in the sample. Finally, the reaction was stopped by adding 2 N H_2SO_4, changing color from blue to yellow, and the absorbance was measured at 450 nm in an microplate reader (Molecular Device, Sunnyvale, CA, USA).

Attachment and invasion assay

Attachment and invasion assays were conducted as previously described (25) with some modifications. Briefly, the KB cells were grown until they reached confluence (1×10^5 cells/well) for assays. For the inhibitory attachment assays of *W. cibaria* on *F. nucleautm*, *W. cibaria* was incubated with the monolayers of KB cells for 1 h, prior to the addition of *F. nucleatum*. The plates were incubated in 5% CO_2 at 37℃ for 1 h and then washed four times with PBS. The monolayers were lysed with distilled water and serially diluted, then plated onto erythromycin-crystal violet agar for *F. nucleatum* (26), and on MRS agar for *W. cibaria*. For the invasion assays, the incubation was continued for 4 h. The monolayers were washed twice with PBS, and fresh medium containing gentamicin (300 μg/ml) and metronidazole (200 μg/ml) were then added. The monolayers were incubated for an additional 1 h to kill the extracellular bacteria. After washing twice with PBS, the cells were lysed with 1 ml of distilled water for 30 min. Internalized

bacteria were enumerated on selective agar plates. The number of adherent bacteria was calculated as the difference between the number of adherent plus internalized bacteria and the number of internalized bacteria. The levels of attachment and invasion were expressed as the percentage of bacteria retrieved following cell lysis relative to the total number of bacteria initially added.

Cytotoxicity

The cytotoxicity of *W. cibaria* was measured using a MTT assay, as described previously (27). The cells were stimulated with live *W. cibaria* at various concentrations (MOI of 10, 100, 1,000) for 24 h. In addition, live *W. cibaria* and *F. nucleatum* were co-cultured with various ratios (0.1:1, 1:1, 10:1). The cultures of the control group were left untreated. MTT solution (5 mg/ml; Sigma) was added to each well culture plate, which was then covered with aluminum foil for 4 h at 37℃ under 5% CO_2. Consecutively, the medium was removed and DMSO (Sigma) was added to extract the MTT formazan. The absorbance of each well was measured at 540 nm using a microplate reader.

Statistics

Each experiment was carried out in triplicate, and the mean value was analyzed further. Statistical analysis was carried out using SPSS version 17.0 (Statistical packages for Social Science version 17.0; SPSS Inc., Chicago, IL, USA). A Mann-Whitney test was used to identify any statistically significant differences in the experiments.

RESULTS

Dose responses of *W. cibaria* to *F. nucleatum*-induced IL-6 and IL-8

To evaluate the dose responses of *W. cibaria* and/or *F. nucleatum* stimulation on epithelial IL-6 or IL-8 gene expression, KB cells were incubated with *W. cibaria* and *F. nucleatum*, either alone or in combination, for 4 h, after which IL-6 and IL-8 mRNA expressed by the cells were examined by RT-PCR. *W. cibaria* inhibited the *F. nucleatum*-induced expression of the IL-6 and IL-8 genes in a dose-dependent manner (Fig. 1A). IL-6 and IL-8 secretion were measured by ELISA to confirm the correlation of mRNA expression with protein production (Fig. 1B). Stimulation of KB cells with *F. nucleatum* for 24 h induced the secretion of both cytokines. Co-culture with *W. cibaria* blocked the secretion of IL-6 induced by *F. nucleatum* in a dose-dependent manner ($p < 0.05$). The release of IL-8 showed a similar pattern to that observed with the release of IL-6 in KB cells stimulated at an *F. nucleatum* to *W. cibaria* ratio of from 1:0.1 to 1:1. However, the inhibitory effect exerted by *W. cibaria* was not shown at an *F. nucleatum* to *W. cibaria* ratio of 1:10.

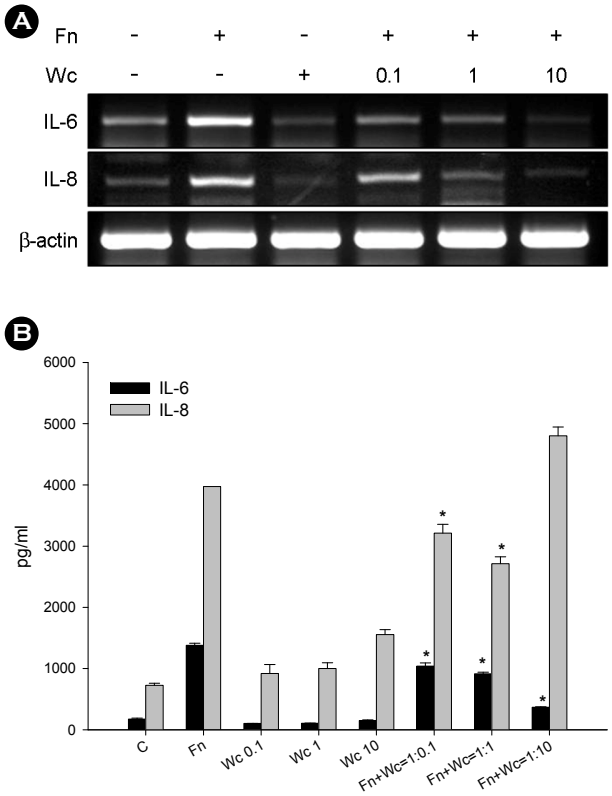

Figure 1. Dose responses of *W. cibaria* on *F. nucleatum*-induced IL-6 and IL-8 production in KB cells. IL-6 and IL-8 mRNA (A) and protein (B) were assayed by RT-PCR and ELISA, respectively. *F. nucleatum* was used at a MOI of 100. *W. cibaria* was used at different MOI, from 10 to 1,000 (which are relatively expressed as 0.1, 1, or 10 *W. cibaria* per *F. nucleatum*). The data is expressed with the mean ± SD of a representative experiment performed in triplicate. C, control cells without stimulation; Fn, cells stimulated with *F. nucleatum*; Wc, cells stimulated with *W. cibaria*; Fn+Wc, *F. nucleatum* with *W. cibaria*. *$p < 0.05$, compared with *F. nucleatum* alone.

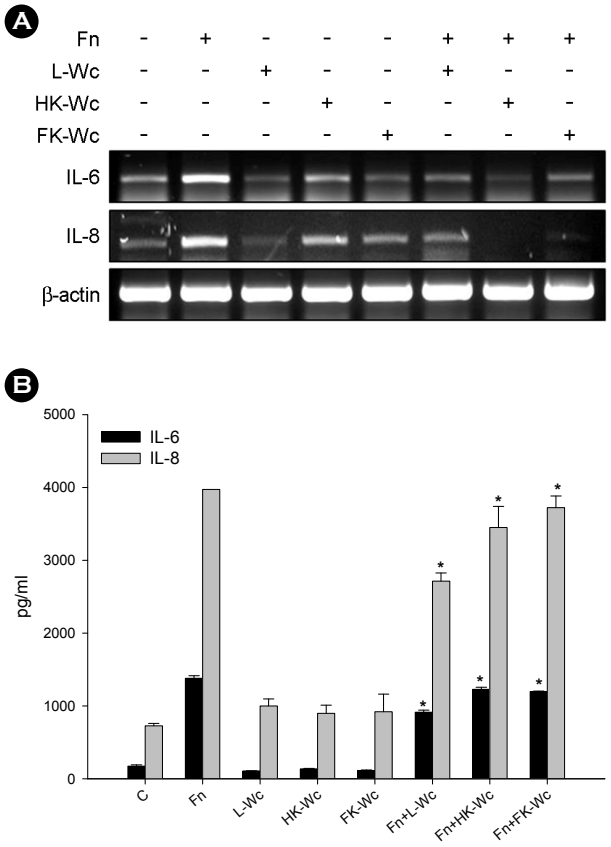

Figure 2. Effects of live and killed *W. cibaria* on *F. nucleatum*-induced IL-6 and IL-8 production in KB cells. IL-6 and IL-8 mRNA (A) and protein (B) were assayed by RT-PCR and ELISA, respectively. KB cells were stimulated at an *F. nucleatum* to live, heat-killed, or formalin-killed *W. cibaria* ratio of 1:1, as described in Materials and Methods. The data is expressed with the mean ± SD of a representative experiment performed in triplicate. C, control cells without stimulation; Fn, *F. nucleatum*; L-Wc, live *W. cibaria*; HK-Wc, heat-killed *W. cibaria*; FK, formalin-killed *W. cibaria*; Fn+L-Wc, *F. nucleatum* with live *W. cibaria*; Fn+HK-Wc, *F. nucleatum* with heat-killed *W. cibaria*; Fn+FK-Wc, *F. nucleatum* with formalin-killed *W. cibaria*. *$p < 0.05$, compared with *F. nucleatum* alone.

Comparative effect between live and killed *W. cibaria* on *F. nucleatum*-induced IL-6 and IL-8

To determine if viable *W. cibaria* were required for the inhibition of *F. nucelatum*-induced IL-6 and IL-8 gene expression, KB cells were incubated with same number of live or killed *W. cibaria* (MOI=100) for 4 h. We found that heat-killed and formalin-killed *W. cibaria* were still able to inhibit *F. nucleatum*-induced expression of the IL-6 and IL-8 genes to a similar extent as live bacteria (Fig. 2A). In addition, all killed *W. cibaria* were significantly able to inhibit *F. nucleatum*-induced IL-6 and IL-8 secretions ($p < 0.05$), however, the inhibitory effect of live *W. cibaria* on *F. nucleatum*-induced IL-6 and IL-8 secretions was higher than that of killed *W. cibaria* (Fig. 2B).

Effect of the separation of *W. cibaria* from host cells on *F. nucleatum*-induced IL-6 and IL-8

To evaluate the effect of direct contact between *W. cibaria* and KB cells on *F. nucleatum*-induced cytokine secretions, *W. cibaria* was separated from KB cells and *F. nucleatum* by tissue culture inserts in the Transwell system. After 24 h incubation, live *W. cibaria* separated from epithelial cells and *F. nucleatum* by a tissue culture insert did not exert any inhibitory effect on IL-6 and IL-8 secretions (Fig. 3A).

Coaggregation effect of *W. cibaria* on *F. nucleatum*-induced IL-6 and IL-8

To investigate the bacterial coaggregation, 1 mM L-canavanine was used to pretreat *F. nucleatum* to inhibit coaggregation. IL-6 and IL-8 response by L-canavanine-treated *F. nucleatum* alone were not different from that of untreated control bacteria. In addition, *W. cibaria* were still able to inhibit L-canavanine-treated *F. nucleatum*-induced IL-6 and IL-8 production independent of bacterial coaggreation (Fig. 3B).

Comparative study of other LAB on *F. nucleatum*-induced IL-6 and IL-8

To investigate the effect of other LAB on *F. nucleatum*-induced cytokine secretions, KB cells were incubated with other LAB strains and *F. nucleatum*, either alone or in combination, for 24 h. All LAB strains were significantly able to inhibit *F. nucleatum*-induced IL-6 secretions. However, the inhibitory effect of *W. cibaria* on *F. nucleatum*-induced IL-6 and IL-8 secretions was higher than that of other LAB including *L. acidophilus*, *L. casei*, *L. fermentum* and *L. reuteri* (Fig. 4).

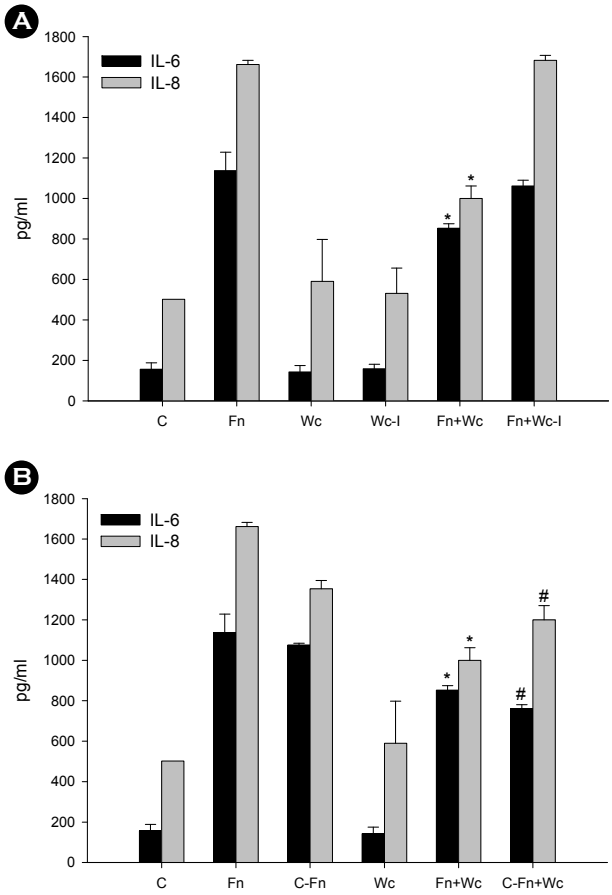

Figure 3. Effect of the separation of *W. cibaria* from host cells (A) and coaggregation effect (B) on *F. nucleatum*-induced IL-6 and IL-8 production in KB cells. IL-6 and IL-8 protein were assayed by ELISA. The data is expressed with the mean ± SD of a representative experiment performed in triplicate. C, control cells without stimulation; Fn, *F. nucleatum*; Wc, *W. cibaria*; Wc-I, *W. cibaria* separated from KB cells through a tissue insert; Fn+Wc, *F. nucleatum* with *W. cibaria*; Fn+Wc-I, *F. nucleatum* with *W. cibaria* separated from KB cells through a tissue insert; C-Fn, L-canavanine pre-treated *F. nucleatum*; C-Fn+Wc, L-canavanine pre-treated *F. nucleatum* with *W. cibaria*. *$p < 0.05$, compared with *F. nucleatum* alone; #$p < 0.05$, compared with L-canavanine pre-treated *F. nucleatum* alone.

Effect of *W. cibaria* on cell attachment and invasion by *F. nucleatum*

Cell attachments were performed to determine if *W. cibaria* affects the cell attachment of *F. nucleatum* to KB cells. *W. cibaria* significantly inhibited the cell attachment of *F. nucleatum* to KB cells, whereas, *F. nucleatum* had no effect on the cell attachment of *W. cibaria* to KB cells (Fig. 5A). Interestingly, both *W. cibaria* and *F. nucleatum* increased the invasion of both bacteria to KB cells (Fig. 5B).

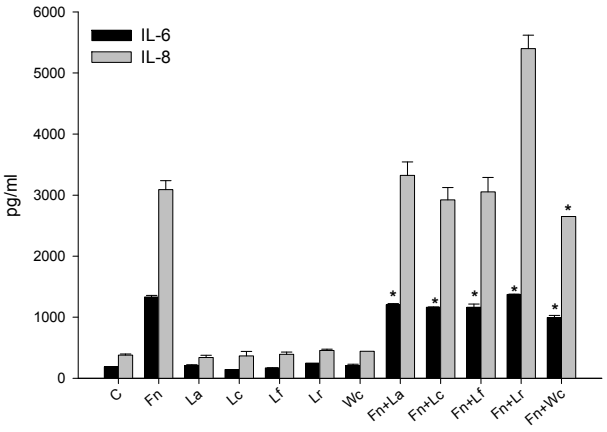

Figure 4. Effects of various lactobacilli on *F. nucleatum*-induced IL-6 and IL-8 production in KB cells. *W. cibaria* and four different *Lactobacillus* species were used, either alone or in combination with *F. nucleatum*, to infect the KB cells. IL-6 and IL-8 protein were assayed by ELISA. The data is expressed with the mean ± SD of a representative experiment performed in triplicate. C, control cells without stimulation; Fn, *F. nucleatum*; La, *L. acidophilus*; Lc, *L. casei*; Lf, *L. fermentum*; Lr, *L. reuteri*; Wc, *W. cibaria*; Fn+La, *F. nucleatum* with *L. acidophilus*; Fn+Lc, *F. nucleatum* with *L. casei*; Fn+Lf, *F. nucleatum* with L. *fermentum*; Fn+Lr, *F. nucleatum* with *L. reuteri*; Fn+Wc, *F. nucleatum* with *W. cibaria*. *$p < 0.05$, compared with *F. nucleatum* alone.

Cytotoxicity of *W. cibaria*

To rule out the possibility that cell toxicity caused by *W. cibaria* infection might have been responsible for the IL-6 and IL-8 inhibition, the viability of the cells was evaluated by MTT assay. No obvious cytotoxic effects were detected at either MOI tested within a 24 h incubation time, and cell viability was ≥ 95% of the untreated control in all experiments (Fig. 6A). In addition, the viabilities of KB cells with co-incubation with *W. cibaria* and *F. nucleatum* were not changed (Fig. 6B).

DISCUSSION

Interactions between the periodontal bacteria and human oral epithelial cells lead to the activation and expression of pro-inflammatory cytokines including IL-6 and IL-8 (28). IL-6 plays a role in host defense, acute phase reactions, immune response, and hematopoiesis. IL-6 has been related to the development of infectious diseases and is involved in both the local and systemic immune response to bacterial

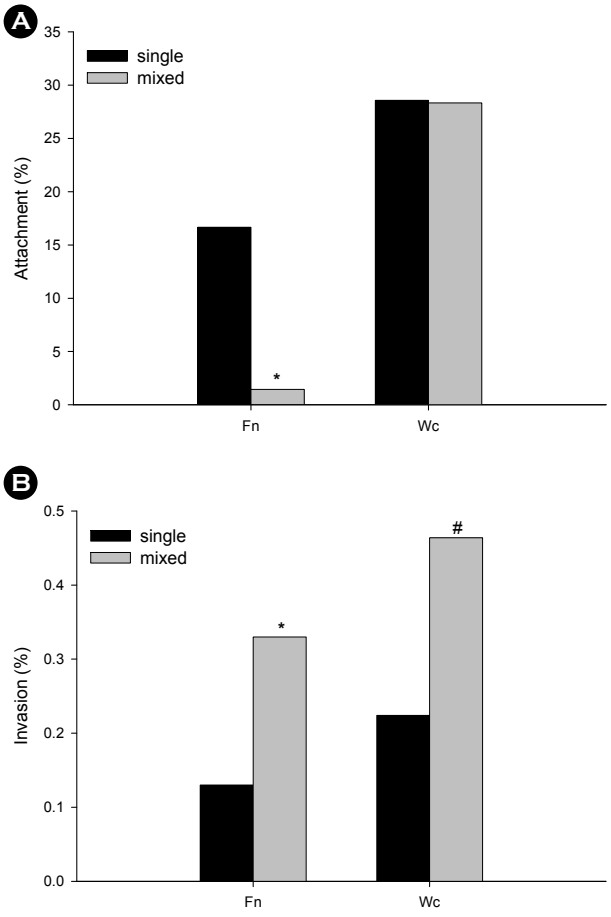

Figure 5. Effect of *W. cibaria* on the attachment (A) and invasion (B) of *F. nucleatum* to KB cells. KB cells were pre-treated with *W. cibaria* for 1 h and stimulated with *F. nucleatum*, as described in Materials and Methods. The levels of attachment and invasion is expressed as a percentage of bacteria retrieved following cell lysis relative to the total number of bacteria initially added. The data is expressed as the mean of a representative experiment performed in triplicate. Fn, *F. nucleatum*; Wc, *W. cibaria*. *$p < 0.05$, compared with *F. nucleatum* alone; #$p < 0.05$, compared with *W. cibaria* alone.

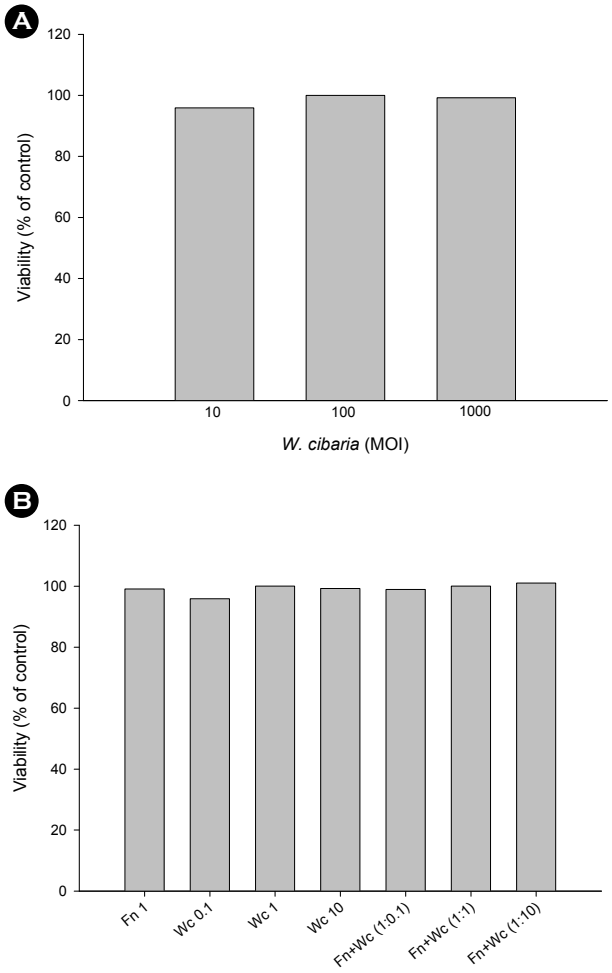

Figure 6. Cytotoxicity of *W. cibaria* on KB cells. (A) KB cells were stimulated with *W. cibaria* at various concentrations (MOI of 10, 100, 1,000) for 24 h. (B) *W. cibaria* and *F. nucleatum* were co-cultured with various concentrations (0.1:1, 1:1, 10:1). Untreated cells were used as a control. The cell viability was assessed using a MTT assay. The data is expressed at the mean of a representative experiment performed in triplicate. Fn 1, *F. nucleatum* was used at a MOI of 100; Wc 0.1, *W. cibaria* was used at a MOI of 10; Wc 1, *W. cibaria* was used at a MOI of 100; Wc 10, *W. cibaria* was used at a MOI of 1000; Fn+Wc (1:0.1), 0.1 *W. cibaria* per *F. nucleatum*; Fn+Wc (1:1), 1 *W. cibaria* per *F. nucleatum*; Fn+Wc (1:10), 10 *W. cibaria* per *F. nucleatum*.

antigens (9). Several reports suggest that cytokines play important roles in the pathogenesis of periodontitis (29, 30). IL-8, a C-X-C chemokine, is up-regulated in oral or gingival epithelial cells after a challenge with several periodontal bacteria, such as *A. actinomycetemcomitans*, *F. nucleatum*, and *P. intermedia* (5, 11, 12). It has been shown that IL-8 was localized in gingival tissue sections of patients with periodontitis, and the levels of IL-8 mRNA were shown to correspond to the severity of periodontitis (31). Periodontal therapy reduces immune cell numbers in the infiltrate and the levels of IL-8 (32), suggesting a relationship between this chemokine and periodontal status.

In the present study, we showed that IL-6 and IL-8 levels in KB cells were up-regulated by *F. nucleatum*. These observations corresponded to the findings that *F. nucleatum* is highly invasive for KB cells and a potent stimulator of IL-8 expression (5). This may be related to the fact that *F. nucleatum* has fimbriae. The presence of fimbriae probably makes *F. nucleatum* adept at adhesion to and internalization

by epithelial cells, during which strong IL-6 and IL-8-inducing stimuli may be generated. Both commensal and pathogenic microorganisms have molecular motifs that can activate pro-inflammatory gene expression, but generally the exposed epithelium can maintain an immune homeostasis in response to its established microbial flora. Diverse mechanisms have been proposed that involve crosstalk between bacteria and epithelial cells to quench inflammation (33). It is a relatively new idea that commensals may also actively contribute to this process.

In the present study, we observed that epithelial exposure to *W. cibaria* decreased IL-6 levels induced by *F. nucleatum* dose dependently. In addition, *W. cibaria* exhibited the capacity to inhibit *F. nucleatum*-induced IL-8 production by epithelial cells. This suggests that *W. cibaria* may contribute to reduce the afflux of inflammatory cells at diseased sites. However, at present, we cannot explain why the high dose *W. cibaria* treatments (MOI of 1,000, *F. nucleatum* to *W. cibaria* ratio of 1:10) increased IL-8. It is possible that very high doses of bacteria or bacterial overgrowth with *W. cibaria* may actually exacerbate inflammation, whereas lower quantities can reduce inflammation. Additional studies will be required to determine whether these effects are important *in vivo*. Similar study was reported that high concentration of live *L. rhamnosus* GG with or without TNF-α resulted in IL-8 production that were greater than those due to TNF-α alone (34).

Certain live commensal and probiotic bacteria have been shown to inhibit IL-8 synthesis induced by TNF-α and pathogenic bacteria in human intestinal epithelial cells (34). By contrast, our results showed that *W. cibari*a did not have to be alive to induce inhibitory effects. Killed *W. cibaria* were still able to inhibit *F. nucleatum*-induced IL-6 and IL-8 levels to a similar extent as live bacteria. However, live *W. cibaria* separated from epithelial cells and *F. nucleatum* by a tissue culture insert did not exert any inhibitory effect. In addition, coaggregation inhibitor did not affect the inhibitory effect of *W. cibaria* on *F. nucleatum*-induced cytokines. Therefore, this inhibitory effect required contact of *W. cibaria* with host cells, but seemed to be independent of bacterial viability and interbacterial coaggregation.

It has been reported that *P. gingivalis* inhibited IL-8 release strongly from gingival epithelial cells, which was partially ascribed to the action of its extracellular proteases (35). Protease activity seemed not to be a factor in our study, because *W. cibaria* did not secrete protease to degrade the IL-6 and IL-8 induced by *F. nucleatum* in a Transwell culture system. The results in our study suggest that direct contact between *W. cibaria* and oral epithelial cells is necessary for the inhibitory effect. Separation of *W. cibaria* from epithelial cells by tissue culture inserts abolished the inhibitory effect on IL-6 and IL-8 production induced by *F. nucleatum* situated in the lower chamber of the culture system. These results suggest that the observed inhibitory effect seemed not to be mediated by secreted soluble factors of *W. cibaria*.

Adherence to epithelial cells is important for colonization. Invasion allows the bacteria not only to evade the host immune surveillance but also to spread into deeper tissues. We recently demonstrated that *W. cibaria* isolates strongly coaggregated with *F. nucleatum* and efficiently adhered to the epithelial cells (21). Therefore, in this study the effects of *W. cibaria* on the adhesion and invasion of *F. nucleatum* to KB epithelial cells were examined. Both of epithelial attachment and invasion by *F. nucleatum* were significantly affected in the presence of *W. cibaria*. However, disruption of interbacterial coaggregation by coaggregation inhibitor did not affect the inhibitory effect of *W. cibaria* on *F. nucleatum*-induced cytokines. These results suggest that the inhibitory effect of *W. cibaria* is not likely to occur through interfering with the interaction between *F. nucleatum* and host epithelial cells.

Recently, probiotics and commensals have been recognized to suppress pathogen-induced pro-inflammatory responses in intestinal models, thus helping to maintain immune homeostasis (15, 16). Often, the inhibitory effect of a probiotic or commensal organism is ascribed to blockade of activator protein 1 and nuclear factor-κB activation (36). It appears that mechanisms vary between different strains and species of bacteria, and between different models. However, the present study focused only the anti-inflammatory activities of *W. cibaria in vitro*.

In conclusion, *W. cibaria* can inhibit the production of IL-6 and IL-8 from human mouth epithelial cells induced by *F. nucleatum*. This suggests that *W. cibaria* is a good candidate for controlling periodontal disease. Further studies on the action mechanisms of this bacterium will be needed to determine their future therapeutic use. Determining the molecular mechanism of action by this may provide new insights into immune homeostasis of the oral epithelium and lead to the development of new therapeutics for mucosal inflammatory diseases.

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