Original Article
Int J Oral Biol 49:1-9, 2024
pISSN: 1226-7155 • eISSN: 2287-6618
https://doi.org/10.11620/IJOB.2024.49.1.1

International Journal of Oral Biology
IJOB
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Fusobacterium nucleatum infection induces CSF3 expression through p38 MAPK and JNK signaling pathways in oral squamous cell carcinoma cells

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Oral bacterial infections substantially affect the development of various periodontal diseases and oral cancers. However, the molecular mechanisms underlying the association between *Fusobacterium nucleatum* (*F. nucleatum*), a major periodontitis (PT)-associated pathogen, and these diseases require extensive research. Previously, our RNA-sequencing analysis identified a few hundred differentially expressed genes in patients with PT and peri-implantitis (PI) than in healthy controls. Thus, in the present study using oral squamous cell carcinoma (OSCC) cells, we aimed to evaluate the effect of *F. nucleatum* infection on genes that are differentially regulated in patients with PT and PI. Human oral squamous cell carcinoma cell lines OSC-2O, HSC-4, and HN22 were used. These cells were infected with *F. nucleatum* at a multiplicity of infection of 100 for 3 hours at 37℃ in 5% CO_2. Gene expression was then measured using reverse-transcription polymerase chain reaction. Among 18 genes tested, the expression of CSF3, an inflammation-related cytokine, was increased by *F. nucleatum* infection. Additionally, *F. nucleatum* infection increased the phosphorylation of AKT, p38 MAPK, and JNK in OSC-20 cells. Treatment with p38 MAPK (SB202190) and JNK (SP600125) inhibitors reduced the enhanced CSF3 expression induced by *F. nucleatum* infection. Overall, this study demonstrated that *F. nucleatum* promotes CSF3 expression in OSCC cells through p38 MAPK and JNK signaling pathways, suggesting that p38 MAPK and JNK inhibitors may help treat *F. nucleatum*-related periodontal diseases by suppressing CSF3 expression.

Keywords: *Fusobacterium nucleatum*, CSF3, p38 mitogen-activated protein kinases, JNK

Introduction

Globally, periodontal disease is characterized by the inflammation and loss of tissues and tooth-supporting structures surrounding the teeth, including the gums and alveolar bones. It is caused by complex interactions among various factors, including pathogenic microorganisms, nutritional deficiencies, smoking, alcohol consumption, and diabetes [1]. Periodontitis (PT) primarily arises as a response to interactions with oral bacteria in the host and has been revealed to affect the oral cavity as well as increase systemic inflammatory responses [2,3]. Several studies have shown that PT is associated with various cancers, including pancreatic, lung, and gastrointestinal cancer [4–6]. Recent research has emphasized the significant

Received January 5, 2024; **Revised** March 4, 2024; **Accepted** March 7, 2024
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and close relationship between PT and oral cancer [7].

Many oral bacteria, such as *Porphyromonas gingivalis* (*P. gingivalis*), *Treponema denticola*, *Tannerella forsythia*, *Prevotella intermedia*, and *Fusobacterium nucleatum* (*F. nucleatum*) cause PT [8]. Among these, *F. nucleatum*, an anaerobic gram-negative bacterium, is also associated with colorectal, and gastrointestinal cancers [9,10]. *F. nucleatum* infection induces DNA damage and chronic inflammation in host cells [11]. It also enhances migration, invasion, and tumorigenesis of oral squamous cell carcinoma (OSCC) cells [12]. OSCC is the most common malignant tumor among head and neck cancers. Lip and oral cavity cancers have been reported to show the 16th highest incidence rate worldwide in 2020 [13]. Despite advances in OSCC-related research, the 5-year survival rate of patients with oral cancer, which is about 50%, has not significantly improved, and the recurrence rate remains relatively high at approximately 30% [14,15]. This highlights the need for novel treatment methods and approaches. Therefore, studying the molecular mechanisms underlying *F. nucleatum* infection is crucial to understand the development of PT and oral cancer.

Granulocyte colony-stimulating factor 3 (CSF3), an inflammation-related cytokine, is a major regulatory factor involved in the survival, proliferation, and differentiation of neutrophils through interaction with the granulocyte colony stimulating factor receptor (G-CSFR) and has been utilized for treating neutropenia [16,17]. CSF3 is upregulated in various human cancers including gastric, lung, and colorectal cancer [18–20]. It serves as a key mediator of the inflammatory responses involved in the proliferation, migration, and apoptosis of cancer cells. In previous studies, high CSF3 expression was found to promote the proliferation, migration, and invasion of glioma cells through STAT3 activation [21]. CSF3 also influences the survival and migration of ovarian cancer cells by activating the JAK2/STAT3 pathway [22]. Furthermore, our previous study demonstrated increased CSF3 expression in patients with PT and peri-implantitis (PI) using RNA-seq [23]. However, the mechanism underlying CSF3 upregulated in the PT and PI remains unclear.

We previously identified several hundred genes that exhibited differential expression in PT and PI samples compared with those in healthy controls. In this study, we examined the molecular mechanisms underlying the differentially expressed genes in the PT and PI and tested the hypothesis that *F. nucleatum* infection could be a contributing factor to the development of these conditions.

Materials and Methods

1. Cell culture

Human OSCC cell lines OSC-20, HSC-4, and HN22 were used in this study, and were maintained in Dulbecco's modified Eagle medium: Nutrient Mixture F-12 (DMEM/F12), Eagle's minimum essential medium, and DMEM, respectively, at 37℃ in 5% CO_2. All cell culture media were supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin.

2. *Fusobacterium nucleatum* culture and infection into OSCC cells

F. nucleatum strain KCTC2640 was cultured in gifu anaerobic medium broth containing vitamin K (5 μg/mL) and hemin (5 μg/mL), in an anaerobic chamber maintained at 37℃ with an atmosphere comprising 90% N_2, 5% H_2, and 5% CO_2. The optical density of 1.0 at 660 nm was equivalent to 10^9 colony-forming units (CFU/mL). For infecting OSCC cells, *F. nucleatum* cells were washed twice and resuspended in phosphate-buffered saline (PBS). The OSCC cells were then infected with *F. nucleatum* at a multiplicity of infection (MOI) of 100 for 3 hours at 37℃ in 5% CO_2. Following infection, the cells were washed twice with PBS and then covered with fresh media containing gentamicin (25 μg/mL). Control cells were subjected to the same media changes and wash conditions, but without bacterial infection.

3. RNA extraction and reverse transcription-quantitative polymerase chain reaction

Total RNA was extracted from OSCC cells using TRIzol reagent (Invitrogen) and quantified using a NanoDrop spectrophotometer (Thermo Scientific); next, 1 μg of total RNA was reverse-transcribed into cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Real-time polymerase chain reaction (PCR) quantification was performed using the TOPreal SYBR Green PCR Kit (Enzynomics) according to the manufacturer's protocol in an ABI 7500 Real-Time PCR Detection System (Applied Biosystems). The primers used for the PCR amplification of mRNAs are listed in Supplementary Table 1. Relative gene expression was determined using the comparative cycle threshold method ($2^{-\Delta\Delta Ct}$) with GAPDH as the control.

4. Western blot analysis

After 24 hours of infection, cells were washed with ice-cold PBS, harvested, and resuspended in buffer A containing 100 mM Tris (pH 8.0), 250 mM NaCl, 1 mM EDTA, and 1% NP-40 with a protease inhibitor cocktail, 10 mM NaF, and 10 mM Na_3VO_4. The protein sample was sonicated and centrifuged at 15,000 rpm for 10 minutes at 4℃. Proteins were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel, and electrophoretically transferred onto a nitrocellulose membrane. The membrane was blocked for 1 hour at room temperature with 5% skim milk in Tris-buffered saline (TBS) containing 0.5% Tween-20 (TBS-T) and incubated with the appropriate primary antibodies diluted in TBS-T (1:1000) at 4℃ overnight. The membrane was washed thrice with TBS-T buffer for 10 minutes each and then incubated with peroxidase-conjugated secondary antibodies diluted in TBS-T (1:5000) for 1 hour at room temperature. After washing with TBS-T thrice for 10 minutes each, the protein bands were visualized using Super-Signal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific) and analyzed using ChemiDoc XRS+ (Bio-Rad). Statistical and densitometric analyses were performed using ImageJ software. The antibodies used in this study were anti-AKT (Cell Signaling Technology), anti-P-AKT (Cell Signaling Technology), anti-p38 (Cell Signaling Technology), anti-P-p38 (Cell Signaling Technology), anti-JNK (Santa Cruz Biotechnology), anti-P-JNK (Santa Cruz Biotechnology), and anti-GAPDH (Santa Cruz Biotechnology). GAPDH was used as a loading control.

5. Inhibitor treatment

The AKT inhibitor (MK2206), p38 MAPK inhibitor (SB202190), and JNK inhibitor (SP600125) were purchased from Sigma-Aldrich, dissolved in dimethyl sulfoxide at a concentration of 10 mM, and stored at –20℃. OSC-20 cells were subjected to MK2206 pre-treatment for 30 minutes prior to infection with *F. nucleatum*, or with SB202190 or SP600125 pre-treatment for 2 hours before infection.

6. Statistical analysis

All experiments were conducted independently, at least in triplicate (n = 3), and the results are expressed as the mean ± standard deviation (SD). Statistical analyses were performed using Student's t-test.

Results

1. *Fusobacterium nucleatum* infection increases CSF3 expression in OSCC cells

Based on our previous study, we confirmed a total of 18 genes that were up- or downregulated in PT and PI using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) [23]. We thus speculated that oral bacterial infections could influence changes in gene expression. Among the 18 genes tested using RT-qPCR, 6 genes were upregulated in 2 cell lines after *F. nucleatum* infection compared with those in the control (Fig. 1A). The most dramatic change was observed in CSF3, which showed a significant increase in OSC-20 and HN22 cells (Fig. 1). Therefore, in subsequent experiments, we focused on understanding the mechanism underlying CSF3 upregulation by *F. nucleatum* infection.

2. *Fusobacterium nucleatum* infection induces phosphorylation of AKT, p38 MAPK, and JNK in OSC-20 cells

F. nucleatum infection is known to activate various signaling pathways such as AKT, p38 MAPK, and JNK in several cell lines, including human gingival fibroblasts, macrophages, and human alveolar epithelial cells [24-26]. To determine whether *F. nucleatum* infection affects these signaling pathways in OSCC cells, we treated OSC-20 cells with *F. nucleatum* at an MOI of 100 and examined the phosphorylation of each protein using western blot analysis. As shown in Fig. 2, infection with *F. nucleatum* led to increased phosphorylation of AKT, p38 MAPK, and JNK. Thus, our findings suggest that *F. nucleatum* infection activates the AKT, p38 MAPK, and JNK signaling pathways in OSC-20 cells.

3. The p38 MAPK and JNK signaling pathways regulate *F. nucleatum*-induced CSF3 expression in OSC-20 cells

To investigate the signaling pathways involved in *F. nucleatum*-induced CSF3 expression, we pretreated OSC-20 cells with the inhibitors of AKT, p38 MAPK, or JNK prior to *F. nucleatum* infection. As shown in Fig. 3, treatment with MK2206, an AKT inhibitor, had no significant effect on CSF3 expression. However, treatment with SB202190 (a p38 inhibitor) and SP600125 (a JNK inhibitor) reduced the expression of *F. nu-*

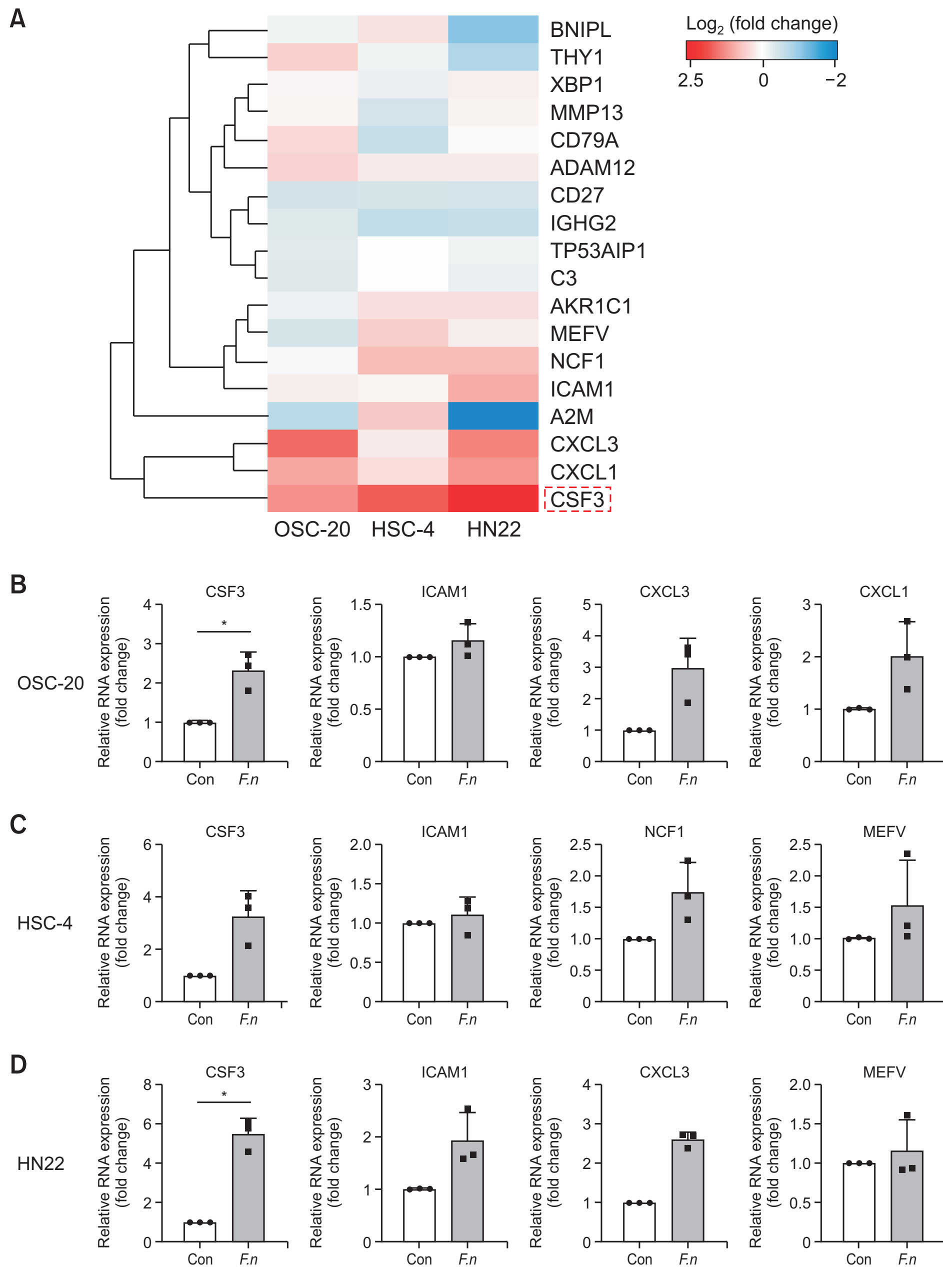

Fig. 1. *Fusobacterium nucleatum* (*F. nucleatum*) infection upregulates several genes that are increased in periodontitis and peri-implantitis. (A) Heapmap showing the gene expression changes in each OSCC cell line upon infection with *F. nucleatum* at a multiplicity of infection (MOI) of 100, using reverse transcription-quantitative polymerase chain reaction. The change was calculated based on the average fold change from triplicate experiments. (B) OSC-20 cells, (C) HSC-4 cells, and (D) HN22 cells were infected with *F. nucleatum* at an MOI of 100 for 3 hours, followed by incubation for an additional 21 hours. Data are represented as the mean ± standard deviation of 3 independent experiments. *p*-values were determined using a two-tailed t-test.
Con, control; *F.n.*, *F. nucleatum*.
*$p < 0.05$.

cleatum-induced CSF3. These data suggest that the increase in CSF3 expression induced by *F. nucleatum* in OSC-20 cells proceeds via the p38 MAPK and JNK signaling pathways.

Discussion

In our previous study, we identified several hundred genes that were differentially expressed in patients with PT and PI than in the healthy controls [23]. Gene ontology analysis showed that many immune-related genes were upregulated in both PT and PI. However, the molecular mechanisms underlying the changes in gene expression remained unclear. In this study, we infected the OSC-20, HSC-4, and HN22 OSCC cell lines with *F. nucleatum* to investigate its influence on the previously reported gene expression. The gene expression changes were examined using RT-qPCR and demonstrated significantly increased CSF3 expression in OSC-20 and HN22 cells (Fig. 1). Notably, previous studies have shown that *F. nucleatum* increases CSF3 expression in human gingival fibroblasts; however, the underlying molecular mechanism has not been clarified [24].

Several studies have shown a correlation between high

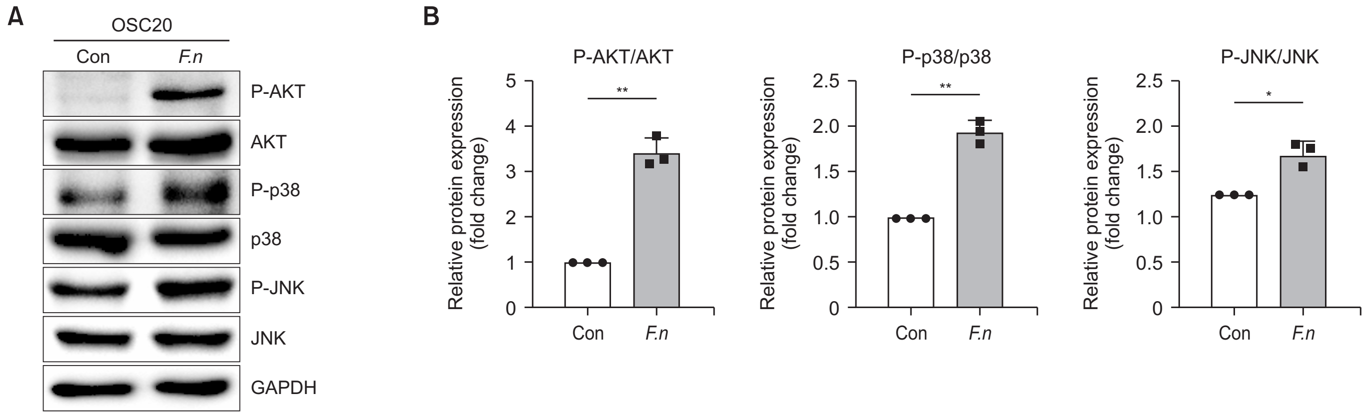

Fig. 2. *Fusobacterium nucleatum* (*F. nucleatum*) infection activates AKT, p38 MAPK, and JNK pathways in OSC-20 cells. (A) OSC-20 cells were infected with *F. nucleatum* at a multiplicity of infection of 100 for 3 hours. Cellular proteins were immunoblotted with antibodies against phosphorylated or total AKT, p38 MAPK and JNK. (B) Based on the triplicate immunoblot images, densitometric analysis was performed for each protein using Image J. The bar graph represents the ratio of phosphorylated protein to the total protein level in control and *F. nucleatum*-infected cells. Data are represented as the mean ± standard deviation from 3 independent experiments. *p*-values were determined using a two-tailed t-test.
Con, control; *F.n.*, *Fusobacterium nucleatum*.
$^{*}p < 0.05$; $^{**}p < 0.01$.

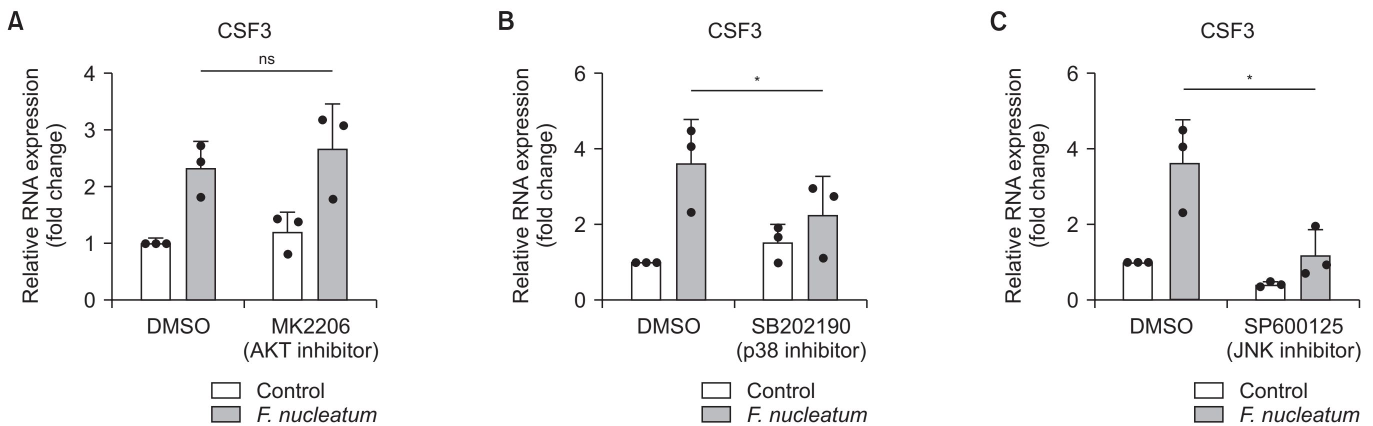

Fig. 3. Inhibitors of p38 MAPK and JNK suppress *Fusobacterium nucleatum* (*F. nucleatum*)-induced CSF3 expression in OSC-20 cells. OSC-20 cells were pre-treated with (A) MK2206 (10 μM) for 30 minutes, (B) SB202190 (10 μM) for 2 hours, or (C) SP600125 (10 μM) for 2 hours, followed by infection with *F. nucleatum* at a multiplicity of infection of 100 for 3 hours and incubation for an additional 21 hours after a phosphate-buffered saline wash. CSF3 expression levels were analyzed using reverse transcription-quantitative polymerase chain reaction. Data are represented as the mean ± standard deviation of 3 independent experiments. *p*-values were determined using a two-tailed t-test.
CSF3, colony-stimulating factor 3; ns, not significant.
$^{*}p < 0.05$.

CSF3 expression and unfavorable outcomes in various cancer, including gastric, lung, and colorectal cancer [18-20]. Furthermore, CSF3 is associated with T-cell regulation, macrophage activation, and ERK signaling pathway activation in colorectal cancer; it also plays a role in the proliferation, migration, and invasion of colorectal cancer cells [27-29]. Increased CSF3 expression has also been observed in oral cancer, but precise mechanistic studies on its initiation and progression in the context of oral cancer are lacking [30].

Previous studies have shown that infection with *F. nucleatum* enhances the phosphorylation of AKT, JNK, and p38 MAPK [24-26]. These signaling pathways are also activated during inflammation and cancer progression [31-33]. Therefore, we examined the activation of AKT, p38 MAPK, and JNK in OSC-20 cells upon *F. nucleatum* infection and observed an increase in the phosphorylation levels of all three pathways compared to those in the control (Fig. 2). To further investigate these pathways, we treated the cells with inhibitors

specific to each pathway and confirmed that CSF3 expression was inhibited by the p38 MAPK inhibitor (SB202190) and JNK inhibitor (SP600125) (Fig. 3). The MAPK signaling pathway regulates various biological processes through multiple cellular mechanisms. p38 MAPK is involved in a broad range of cellular processes, including cell proliferation, differentiation, survival, and apoptosis, and plays a crucial role in activating immune responses by regulating the production of inflammatory cytokines such as tumor necrosis factor α, and interleukin (IL)-1β [34,35]. JNK, known as a stress-activated protein kinase, is activated by various stimuli such as cytokines or bacterial infections. It also participates in cell proliferation, survival, migration, apoptosis, and cell cycle regulation and promotes tumor development in various cancers [36,37]. Therefore, therapies targeting p38 MAPK and JNK are being investigated for cancer treatment [32,38]. Our results suggest that p38 MAPK and JNK inhibitors could be used as potential therapeutic agents in patients with PT and PI.

In our previous study, CSF3 expression increased by approximately 600-fold in patients with PT compared with that in healthy individuals, a value much higher than the 3-fold increase observed upon *F. nucleatum* infection in OSC-20 cells [23]. This discrepancy could be attributed to the difference between chronic inflammation and acute infection. Periodontal disease is associated with chronic inflammation that affects the tissues surrounding and supporting the teeth [39]. Various bacterial species within the oral cavity interact closely with each other; however, an imbalance between the bacterial communities and the host, results in inflammation [3,40]. Our experiment, which involved a single infection with *F. nucleatum* for 3 hours, only mimicked acute inflammation. Therefore, additional research is needed to investigate co-infection with other oral pathogens as well as prolonged infection.

Since what we used in our experiment is *F. nucleatum* itself, it is unclear whether the increase in CSF3 due to *F. nucleatum* is a direct effect. According to Yin and Dale [41], it was known that CSF3 increased due to treatment with extract of *F. nucleatum* cell wall. Therefore, it suggests that *F. nucleatum* cell wall plays an important role in enhancing CSF3, and this increase is a direct effect of *F. nucleatum* infection.

There was report that proliferation of Tca8113 tongue squamous cell carcinoma cells increase due to *F. nucleatum* infection [42]. In addition, it was observed that *F. nucleatum* infection increases migration of OSCC cells such as SCC-9 and HSC4 [43]. Furthermore, *F. nucleatum* infection increases the expression of cytokines such as GM-CSF, CXCL1, and IL-8 in pancreatic cancer cells such as BxPC3, Panc1, HPAC, and Capan1, and these cytokines enhance proliferation and migration of cancer cells [44]. Increased levels of CSF3 have been found to enhance proliferation and migration of cells in glioma, human epithelial ovarian cancer, and colon cancer cell lines [21,22,28]. Considering these findings collectively, it can be speculated that the influence of CSF3 on proliferation and migration of cancer cells increased by *F. nucleatum* infection.

The association between oral bacterial infection and carcinogenesis of OSCC has been well studied. Recent research has highlighted a notable correlation between *P. gingivalis* infection and pancreatic cancer [45]. *P. gingivalis* infection is known to increase oncogenic phenotypes such as alterations in apoptosis and the cell cycle [46]. *P. gingivalis* upregulates IL-8 and MMPs, leading to increased invasiveness of oral cancer cells [47]. Furthermore, prolonged exposure to *P. gingivalis* has been reported to enhance the aggressiveness of oral cancer cells [48]. In addition, multiple studies have underscored a robust link between *F. nucleatum* and colorectal cancer [9]. These studies suggest that enhanced oral hygiene and effective treatment of periodontal disease may also be beneficial in inhibiting cancer progression.

In conclusion, our study demonstrated that *F. nucleatum* infection can increase CSF3 expression in OSC-20 cells by regulating the p38 MAPK and JNK signaling pathways. These data suggest that p38 and JNK inhibitors can be used as potential therapeutic agents for *F. nucleatum*-related periodontal diseases.

Funding

This work was supported by a 2-year Research Grant of Pusan National University.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

Supplementary Data

Supplementary data is available at http://www.kijob.or.kr only.

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