Involvement of *Fusobacterium Nucleatum* in Bone Resorption and Periodontal Tissue Inflammation

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Abstract

Fusobacterium nucleatum is a gram-negative anaerobic bacterium that is resident in the oral cavity and causes various infectious diseases including periodontal disease and has recently been linked to colorectal cancer. In this study, we investigated the immune response of inflammatory periodontal tissue in mice to elucidate how *F. nucleatum* affects the immune responses of oral immune system. *F. nucleatum* was suspended in 5% carboxymethyl cellulose (CMC), and mice were inoculated intraorally 5 times for 3 weeks. Mononuclear cells were isolated from gingival tissues 1 and 30 days after the last oral inoculation, followed by flow cytometry using fluorescently labeled antibodies, DNA extraction, and quantitative PCR. The histopathological examination of gingiva was performed with hematoxylin and eosin staining. The mandibles were collected and the alveolar bone resorption was measured using micro CT. In the group inoculated with *F. nucleatum*, infiltration of inflammatory cells was observed under the gingival mucosa, and remarkable horizontal bone resorption of alveolar bone was observed. In addition, real-time PCR analysis revealed that pro- interleukin (IL)-18 expression and receptor activator of nuclear factor kappa-B ligand (RANKL)/ osteoprotegerin (OPG) ratio increased in gingival tissues. Furthermore, flow cytometry analysis confirmed that CD4⁺RANKL⁺ cells gradually increased in the gingival inflammation lesion. These results suggested that oral inoculation of *F. nucleatum* caused inflammation of the gingival tissue and activated osteoclasts via CD4⁺RANKL⁺ cells, resulting in alveolar bone resorption.

Keywords :

inflammation, bone resorption,
oral infection,
Fusobacterium nucleatum

Introduction

Periodontal disease is a chronic inflammatory disease that affects many adults. It is also a major cause of tooth loss and is characterized by chronic infection by gram-negative anaerobic bacteria in dental biofilms. It leads to irreversible destruction of the tissues that support the teeth and can be detected clinically by loss of periodontal pockets and alveolar bone (1, 2). Periodontitis often causes bacteremia, and the relationship between periodontitis and systemic diseases caused by periodontal

pathogens has been investigated (3). Recently, it has been suggested that intestinal dysbiosis due to periodontal pathogens is a potential cause of various diseases (4).

Periodontitis is a multifactorial disease, and the genetic background of the patient, the presence of pathogenic bacteria and immune mechanisms are important factors. During the process of inflammation in response to the plaque bacterial species, gingival fibroblasts produce interleukin (IL)-1 and IL-18 (5). Cytokine secretion at the site of inflammation is initially protective in the removal of infectious bacteria, but excessive or persistent pro-inflammatory cytokine production is associated with periodontal destruction, accompanied by loss of periodontal

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attachment and alveolar bone resorption caused by receptor activator of nuclear factor kappa-B ligand (RANKL)/ osteoprotegerin(OPG) signaling pathway. Gingival crevicular fluid(GCF) and salivary cytokine levels are higher in patients with aggressive periodontitis than in healthy individuals and decrease after periodontal treatment(6). Macrophages are an important source of inflammatory cytokines such as IL-1 β and TNF- α . In pathogenic dysregulation, these cytokines contribute to host tissue destruction. Therefore, understanding the mechanism of immune cell signaling induced by periodontal pathogens can provide useful information for the prevention and treatment of periodontitis.

F. nucleatum is a gram-negative anaerobic gonococcus resident in the oral cavity, which causes various infections including periodontal disease, as well as being associated with GI disorders such as colorectal cancer and inflammatory bowel disease and adverse pregnancy(7). The bacteria also associated with atherosclerosis, respiratory tract infections, organ abscesses, rheumatoid arthritis, and Alzheimer's disease(7). In oral diseases, *F. nucleatum* is associated with a variety of periodontal diseases, including gingivitis and advanced chronic periodontitis, periodontitis including localized aggressive periodontitis and systemic aggressive periodontitis(8). It increases with disease severity, and rate of progress in inflammation and depth of pocket(8).

F. nucleatum has been reported to produce inflammatory cytokines such as IL-1 and IL-6 in gingival epithelial cells and macrophages in vitro(9, 10).

However, there is no report that links the inflammation caused by *F. nucleatum* and the pathology of periodontal disease. Therefore, in this study, we investigated the effects of oral *F. nucleatum* infection on bone resorption and periodontal inflammation in mice.

Materials and Methods

Bacterial Strain

F. nucleatum (ATCC 23726) was cultured on anaerobic blood agar plates (Becton Dickinson, Franklin Lakes, NJ, USA) in a model 1024 anaerobic system (Forma Scientific, Marietta, OH, USA) with 10% H₂, 80% N₂, and 10% CO₂ for 3–5 days. Cultures were then inoculated into brain-heart infusion (Difco Laboratories, Detroit, MI, USA) supplemented with 5% fetal bovine serum for 2 days until OD_{660nm}=0.8 was reached, corresponding to 10⁹ CFU/mL.

The cultured cells were centrifuged at 8000 $\times g$ for 20 min at 4°C and resuspended in 5% carboxymethyl cellulose (CMC) for oral infection.

Mice

Eight-week-old female BALB/c Cr Slc (BALB/c) mice, obtained from Sankyo Laboratories (Tokyo, Japan), were provided regular mouse feed and water *ad libitum*. The mice were maintained under specific-pathogen-free conditions on temperature-controlled clean racks with a 12-h light-dark cycle. All animal experiments were performed in accordance with the guidelines of the Bioscience Committee of Nihon University and were approved by the Institutional Animal Care and Use Committee of Nihon University (Approval number: AP19MAS006-1).

Oral infection

Mice were randomly divided into two groups (n=8 per group). The first group was orally challenged with live *F. nucleatum* (1 $\times 10^9$ CFU/100 μ L with 5% CMC/mouse) once per day for 15 days. The second group consisted of sham-infected mice that received 100 μ L of 5% CMC.

Histological analysis of gingival tissue and the measurement of alveolar bone loss

Thirty days after the last infection, mice were sacrificed and the skin of the lower jaw was removed and fixed in 4% paraformaldehyde in PBS for 24 h. The lower jaw was decalcified by incubation in 150 mM EDTA in PBS for 5–7 days at 4°C and then embedded in paraffin. Four-micrometer-thick serial sections were then prepared and stained with hematoxylin and eosin. Horizontal bone loss around the maxillary molars was assessed using a morphometric method, as described previously(11). Briefly, after removing gingival tissue, skulls were immersed overnight in 3% hydrogen peroxide, pulsed for 1 min in bleach, and stained with 1% methylene blue. The distance from the cemento-enamel junction (CEJ) to the alveolar bone crest (ABC) was measured at 14 buccal sites per mouse(12). Measurements were made under a dissecting microscope ($\times 20$) fitted with a video image marker measurement system standardized to provide measurements in millimeters.

GMC isolation

At 1, 7, 15, and 30 days after the last *F. nucleatum* in-

fection, mice were sacrificed and gingival tissues from the upper and lower jaws were carefully removed using microsurgical tweezers under a stereomicroscope. Cells from gingival tissues were prepared by gently teasing the tissue using sterile stainless steel screens, followed by an enzymatic dissociation procedure with 0.3 mg/mL of collagenase (Nitta Gelatin Co. Ltd., Osaka, Japan) in RPMI 1640 (Wako Pure Chemical Industries Ltd., Osaka, Japan) (13). GMCs were purified on discontinuous Percoll gradients (Pharmacia Fine Chemicals, Uppsala, Sweden) and resuspended in RPMI 1640 supplemented with HEPES buffer (15 mM), L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 µg/mL), and 10% fetal bovine serum (Biofill, Victoria, Australia) (complete medium). GMC-enriched populations (2×10^5 cells) were stained with a combination of fluorescence-conjugated or biotinylated monoclonal antibodies, including anti-CD4, and -RANKL (BD Pharmingen, San Diego, CA, USA). The samples were subjected to flow cytometry analysis (FACSCalibur™; BD Biosciences).

Analysis of gene expression in GMCs

At 1 and 30 days after the last *F. nucleatum* infection, total RNA from gingival tissue samples was extracted using a RNeasy Mini kit and treated with DNase I (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions. Aliquots of RNA were then reverse-transcribed with oligo(dT) primers using SuperScript® reverse transcriptase (Invitrogen Corp., Tokyo, Japan) to generate cDNA. Quantitative real-time RT-PCR analyses were performed using a Thermal Cycler Dice real-time PCR system (Takara Bio Inc., Otsu, Japan) in accordance with the manufacturer's protocol. All reactions were carried out in a total volume of 25 µL, containing 30 ng of reverse-transcribed cDNA, 12.5 µL of 2x SYBR Green PCR Master Mix (Takara Bio Inc.), and each primer at 100 nM. The primer sequences were as follows:

pro-IL-1p forward (5'-TGACTGACTACGCCTGCCTG-3') and
reverse (5'-TCTTTGAAGTTGACGGACCC-3');
pro-IL-18 forward (5'-CAGGCCTGACATGTTGTGCAA-3') and
reverse (5'-TCTGACATGGCAGCCATTGT-3');
RANKL forward (5'-CAGGTCCAGCGCAATATAA-3') and
reverse (5'-CTGGACATGTGCCACTGAGAA-3');

OPG forward (5'-AAACAGCCCGTGACCTTCCTA-3') and

reverse (5'-AGGTTGGTGCACAGCTTCACAA-3');

GAPDH forward (5'-TGTGTCCGTCGTGGATCTGA-3') and

reverse (5'-TTGCTGTTGAAGTCGCAGGAG-3').

PCR was performed in duplicate for each gene. Target mRNA levels were normalized to that of GAPDH. PCR was performed using the following protocol: 95°C for 30 s, followed by 50 cycles of 95°C for 5 s, 60°C for 30 s. Next, a dissociation curve analysis was performed to confirm specificity. The amplification of each gene and melting curve analysis were performed in triplicate. Target mRNA levels were normalized to that of GAPDH mRNA.

Statistical analysis

All results are presented as means $\pm$ the standard errors of the mean (SEM), and experimental groups were compared with controls using an unpaired non-parametric Mann-Whitney U test in Statview software.

Result

F. nucleatum-challenge induces gingival inflammation and alveolar bone loss

Oral infection of BALB/c mice with *F. nucleatum* induced inflammatory cell infiltration and connective tissue thickening compared to the control group treated with CMC on 30 days after the final administration (Fig. 1A). In the infected group, non-thinning of the alveolar bone was also observed. Furthermore, in the measurement of alveolar bone loss, the distance from CEJ to ABC in 14 locations of the upper jaw was measured, and the average value of the total values for each mouse was shown. The distance of the control group was 213 ± 15.0 µm, while the average value of the *F. nucleatum* infected group was 329 ± 11.9 µm. Therefore, oral infection with *F. nucleatum* induced significant bone resorption of alveolar bone compared to the control group on 30 days after the final administration (Fig. 1B and C).

F. nucleatum-challenge downregulates the expression of pro-inflammatory- and bone resorption-related factors

Inflammatory effects on bone resorption are mediated by inflammatory cytokines (14), and RANKL and OPG are involved in osteoclastogenesis (14).

Therefore, we next examined the expression of gingi-

Fig. 1. *F. nucleatum*-induced gingival inflammation and alveolar bone loss. (A) Histochemical analysis of gingival tissue. Thirty days after the last infection with *F. nucleatum*, mouse lower jaws with gingival tissue were stained with hematoxylin and eosin. (B) Thirty days after the last infection, the distance from the cemento-enamel junction (CEJ) to the alveolar bone crest (ABC) at 14 predetermined sites in the defleshed maxilla were measured and totaled for each mouse. (C) Bone measurements were performed a total of three times by two evaluators using a random and blinded protocol. All values are presented as the means $\pm$ SEM of six mice per group; * $p < 0.05$.

Fig. 2. Gene expression of pro-inflammatory- and bone resorption-related molecules in mouse gingival tissue following *F. nucleatum* challenge. Relative mRNA levels normalized to GAPDH were determined with real-time RT-PCR. Data are expressed as fold-change in mRNA levels compared to controls. All values are presented as the means $\pm$ SEM of six mice per group; * $p < 0.05$.

val inflammatory cytokine genes and the ratio of RANKL/OPG gene expression. Oral infection of BALB/c mice with *F. nucleatum* showed increased expression of pro-IL-18 in the gingiva compared with the control group treated with CMC on 30 days after the final administration. We also observed a significant increase in the RANKL/OPG ratio due to *F. nucleatum* infection (Fig. 2A and B). On the other hand, oral infection with *F. nuclea-*

tum did not significantly increase the expression of pro-IL-1 β in the gingiva compared to the control group treated with CMC.

F. nucleatum-challenge induce $CD4^+RANKL^+T$ cells in GMC

T cells play a central role in regulating immune responses, and many T cells are infiltrated in periodontitis

Fig. 3. RANKL expression by CD4⁺T cells in mice orally infected by *F. nucleatum*.

Thirty days after the last infection with *F. nucleatum*, the frequency of RANKL⁺CD4⁺ T cells was examined by FACS CaliburTM. All values are presented as the means $\pm$ SEM of six mice per group; * $p < 0.05$.

tissues. Since RANKL, an osteoclast differentiation factor, has been confirmed to be produced in immune cells by inflammatory reactions(15), we next examined the increase or decrease in the ratio of CD4⁺RANKL⁺T cells in GMC. Oral infection of BALB/c mice with *F. nucleatum* showed a significant increase ($p < 0.05$) in the ratio of CD4⁺RANKL⁺T cells in GMC compared to the control group treated with CMC on 30 days after the last administration (Fig. 3).

Discussion

Periodontal disease is a chronic inflammatory disease caused by infection with anaerobic bacteria in the oral cavity, and a disease which bacteria in the biofilm cause inflammation in the gums and eventually dissolve the jaw bone that supports the teeth. *F. nucleatum* is one of the periodontopathic bacteria and plays a central role in dental plaque formation, and forms a biofilm by coaggregating with other bacteria(16).

In this study, *F. nucleatum* challenge induced gingival inflammation and alveolar bone loss. Inflammatory cytokines have been shown to play an important role in the regulation of inflammatory bone resorption. It has been reported that IL-1, IL-6 and TNF- α promote bone resorption, while IFN- γ and IL-4 suppress(14, 17). Further, one of the pattern recognition receptors, NLR family, pyrin domain containing 3 (NLRP3) forms a complex called NLRP3 and induces the production of IL-1 β and IL-18, which are inflammatory cytokines. Both processing

and pyroptosis of IL-1 β and IL-18 precursor induced by inflammasome activation, are deeply involved in immune system activation and defense against infection(18), and play an important role in maintaining periodontal tissue homeostasis. On the other hand, their excessive activation leads to destruction of periodontal tissue(19).

Our results indicated that oral infection of mice with *F. nucleatum* increased expression of pro-IL-18 in the gingiva. However, the expression of pro-IL-1 β , a precursor of IL-1 β , did not increase with *F. nucleatum* infection. In future, it will be necessary to search for changes in IL-1 β expression over time and to search for trends in other inflammatory cytokines such as IL-6 and TNF- α .

RANKL is a member of the TNF superfamily and is expressed on the surface of osteoblasts and binds to the receptor activator of NF κ B (RANK) on osteoclast precursor cells. The binding of RANK to RANKL activates a transcription factor downstream of TNF receptor associated factor 6 (TRAF6) which is adapter protein of RANK, and osteoclast differentiation proceeds(20). Osteoprotegerin (OPG) acts as a decoy receptor for RANKL and suppresses osteoclast formation(21). In our experiment, oral infection of mice with *F. nucleatum* increased the ratio of RANKL/OPG genes in the gingiva. In fact, *F. nucleatum* infection increased RANKL gene expression and decreased OPG gene expression (data not shown). This suggests that the increase in RANKL is deeply involved in osteoclast formation while OPG acting to suppress osteoclast formation is decreased.

RANKL has also been confirmed to be produced not only by mesenchymal cells such as osteoblasts and fibroblasts, but also by immune cells such as T cells and B cells by inflammatory reactions(4, 22). Thus, it has become clear that RANKL plays an important role in inflammatory bone resorption. In our results, oral infection of mice with *F. nucleatum* induced an increase in CD4⁺RANKL⁺T cells in GMC. In periodontal tissues that induced LPS-induced bone resorption, infiltration of T cells and RANKL positive cells were confirmed, and these increases were observed as bone resorption increased(23). It has also been confirmed that T cells infiltrating human periodontal tissues expressed RANKL(24). These results suggest that in periodontal tissues, T cells may be involved in promoting bone resorption at the lesion site.

In conclusion, our results have shown that oral admin-

istration of *F. nucleatum* induces gingival inflammation and alveolar bone loss. These results suggest that *F. nucleatum* causes gingival inflammation through activation of inflammasome, activates osteoclasts through the increase of CD4⁺RANKL⁺T cells, and leads to alveolar bone resorption.

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