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The role of invasion ability and proteolytic activity of *Porphyromonas gingivalis* in the murine model of periodontitis

치주염 생쥐 모델에서 *Porphyromonas gingivalis*의

침투 능력과 단백질 분해 능력의 역할

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백 금 진

Abstract

The role of invasion ability and proteolytic activity of *Porphyromonas gingivalis* in the murine model of periodontitis

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Background

Periodontitis is a polymicrobial infectious disease caused by several plaque-associated bacteria, including *Porphyromonas gingivalis, Treponema denticola, and Tannerella forsythia*. *P. gingivalis,* a gram-negative obligate anaerobe, has

a number of virulence factors such as cysteine proteases (gingipains), LPS, capsule, and fimbriae. Among them, gingipains have been described as a major virulence factor to evade the host immune defense by disrupting cytokine signaling network. Gingipains can degrade a number of host proteins, including cytokines/chemokines, and complement proteins. *P. gingivalis* has been shown to invade various host cells *in vitro*, including endothelial cells, gingival fibroblasts, and gingival epithelial cells. *P. gingivalis* has an inter-strain variability in both the proteolytic activity and invasion ability.

Gingival epithelia provide the chemical, physical, and immunological barriers against the invading bacteria. The physical barrier is regulated with the expression of tight-junction (TJ) proteins such as junctional adhesion molecules (JAMs), zonula occludens (ZOs), and claudins. *P. gingivalis* can modulate the expression of epithelial junctional proteins. In addition, the impairment of epithelial physical barrier is involved in the pathogenesis of periodontitis.

The role of invasion ability and proteolytic activity of *P. gingivalis* in the pathogenesis of periodontitis has not been studied at the same time. The aims of this study were to determine the effect of the invasion ability and proteolytic activity of *P. gingivalis* on the development of periodontitis and the direction of immune response in mice using *P. gingivalis* clinical isolates with different invasion ability and proteolytic activity.

Methods

Three clinical isolates of *P. gingivalis* (P1, P4, and P8) obtained from the diseased sites of periodontitis patients were used in the present study (P1: low invasion ability and low proteolytic activity, P4: low invasion ability and high proteolytic activity, P8: high invasion ability and low proteolytic activity).

To examine the effect of *P. gingivalis* clinical isolates with different invasion ability and proteolytic activity on the epithelial physical barrier function, the transepithelial electrical resistance (TER) of immortalized human gingival keratinocyte HOK-16B infected with *P. gingivalis* clinical isolates was measured. In addition, the levels of the TJ proteins (JAM-1 and ZO-1) were measured by immunofluorescence microscopy.

To determine the role of *P. gingivalis* gingipain in the bacterial invasion and the regulation of epithelial physical barrier function, the invasion of *P. gingivalis* into HOK-16B cells was measured by a flow cytometric invasion assay using *P. gingivalis* ATCC 33277 (wild-type) and isogenic gingipain deletion mutant [KDP 129 (*kgp*-), KDP 133 (*rgpA*$^{-}$ *rgpB*$^{-}$) and KDP 136 (*kgp*$^{-}$ *rgpA*$^{-}$ *rgpB*$^{-}$)]. Additionally, the effect of wild-type and mutants on TER of HOK-16B cells was measured.

To examine the role of invasion ability and proteolytic activity of *P. gingivalis* in the development of periodontitis in mice, the alveolar bone loss was measured four weeks after oral inoculation (six times, 2 day apart) of *P.*

gingivalis (10^9 cells/mouse). To detect the bacteria within the gingival tissues after *P. gingivalis* inoculation, digoxigenin-labeled *P. gingivalis*-specific probe was prepared and then *P. gingivalis* was detected in paraffin-embedded tissue sections using *in-situ* hybridization. The presence of neutrophils or T cells in tissue sections were also determined by immunohistochemistry using specific antibody to Ly6G or CD3. To investigate the direction of adaptive immune response by *P. gingivalis* infection in mice, the levels of *P. gingivalis*-specific IgG2a, IgG1, and IgA antibodies in sera were measured by ELISA. The levels of IFN-γ, IL-4, and TGF-β1 proteins in mice cervical lymph node lysates was measured by ELISA.

Results

The TER of HOK-16B cells was decreased by all *P. gingivalis* strain and an inter-strain difference was observed between P1 and P4 at 8 hours. The levels of TJ proteins (JAM-A and ZO-1) in HOK-16B cells was decreased by *P. gingivalis* clinical isolates, and P4 strain had the greatest effects. The invasion ability of gingipain mutant KDP 136 strain was significantly decreased compared with those of wild-type strain (ATCC 33277). Wild-type and KDP 133 strain remarkably decreased the TER of HOK-16B cells.

When experimental periodontitis was induced using P1, P4, or P8 strain in mice, P4 strain induced higher levels of alveolar bone loss than either P1 or P8 strain. The invasion levels of P4 strain into the gingival tissues were higher than

those of either P1 or P8 strain but statistical significance was not reached. Different from expectation, the number of neutrophils and T cells detected within the gingival tissues was not increased by *P. gingivalis*. The levels of anti-*P. gingivalis* IgG2a and IgG1 were not different among groups. However, the levels of anti-*P. gingivalis* IgA against P1 were strongly induced. Interestingly, the levels of IFN-γ, IL-4, and TGF-β1 were decreased in cervical lymph node of all *P. gingivalis*-infected group compared with sham control.

Conclusion

P. gingivalis clinical isolates impaired epithelial barrier function through the regulation of TJ protein expression. The proteolytic activity, but not invasion ability, of *P. gingivalis* may play an important role in the pathogenesis of murine periodontitis model.

Keywords: Invasion ability, Proteolytic activity, Physical barrier, *Porphyromonas gingivalis*, Pathogenesis, Periodontitis

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CONTENTS

1. Introduction

Periodontitis is a chronic inflammatory disease that results from the interactions between subgingival bacteria and host immune responses. The disease can lead to the alveolar bone loss, tissue destruction, and tooth loss.[1] Among the subgingival bacteria harbored in subgingival biofilm, red-complex bacteria including *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia* have been strongly associated with chronic periodontitis.[2]

P. gingivalis has a variety of virulence factors such as capsule, LPS, major fimbriae, and cysteine proteinases (gingipains) that have been reported to contribute to the pathogenicity of *P. gingivalis*. [3] Among these factors, gingipains, which are composed of arginine-specific (Rgp; RgpA and RgpB) and lysine-specific (Kgp) gingipain based on their substrate specificity, can hydrolyze various host proteins, including immunoglobulin, complement proteins, collagens, fibronectin, cell receptors, and cytokine/chemokine.[4,5] The degradation of various cytokines, including interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-4, IL-5, IL-6, IL-10 and IL-17[6-11], suggests that *P. gingivalis* may be able to modulate the host immune response for its benefit by disrupting the cytokine signaling network.

Some bacteria avoid phagocytic clearance and humoral immunity by invading host cells.[12,13] *P. gingivalis* has the ability to invade various host cells, including endothelial cells, gingival fibroblast, and gingival epithelial cells.[14-

[17] Among the virulence factors of *P. gingivalis*, major fimbriae (FimA) and gingipains have been involved in the adhesion to and invasion into the host cells.[18-21] In addition, the presence of *P. gingivalis* within the gingival tissues obtained from periodontitis patients has been reported,[22,23] suggesting an important role of bacterial invasion in the pathogenesis of periodontitis.

The gingival epithelia form physical, chemical, and immunological barriers against invading plaque-associated bacteria, and they provide the first line of host defense. Gingival epithelial cells are connected to each other by protein complexes consisting of cytoskeletal, cytosolic, and transmembrane proteins. Among these proteins, zonula occludens (ZO), junctional adhesion molecules (JAMs), occludin, and claudins assemble-tight junction (TJ) structures that regulate the transport of ions, solutes, and water through the paracellular pathway.[24,25] The function of the physical barrier is modulated with the expression and localization of TJ proteins.[26] *P. gingivalis* can modulate the expression of epithelial junctional proteins such as occludin and E-cadherin that involves gingipains.[27,28] Additionally, the pathogenesis of periodontitis involves the disruption of physical barrier.[29]

P. gingivalis has an inter-strain variability in both proteolytic activity and invasion ability.[6,16] The virulence factors of *P. gingivalis* and its pathogenicity have been studied using experimental animal models. In a mouse abscess model, the heterogenic virulence of *P. gingivalis* has been associated with the invasion and proteolytic activities of *P. gingivalis* clinical isolates with type II fimbriae.[30]

In addition, Kgp and RgpB gingipain are more important than RgpA for virulence of *P. gingivalis* in a periodontitis mouse model.[31] This author previously reported that the invasion ability, but not the cytokine proteolytic activities, of *P. gingivalis* exhibited a strong positive correlation with the clinical parameters of subjects who harbored the isolates.[6]

The aims of this study were to identify the effects of the invasion ability and proteolytic activity of *P. gingivalis* on the development of periodontitis and to determine the direction of immune response in mice using *P. gingivalis* clinical isolates with different invasion abilities and proteolytic activities. Three clinical isolates of *P. gingivalis* (P1, P4, and P8) were obtained from three patients with periodontitis for present study. *P. gingivalis* clinical isolates have different proteolytic activity and invasion ability (P1: low proteolytic activity and low invasion ability, P4: high proteolytic activity and low invasion ability, P8: low proteolytic activity and high invasion ability)[6].

2. Materials and Methods

2.1. Cell culture

Immortalized human gingival keratinocyte HOK-16B cells were maintained in keratinocyte growth medium (KGM; Clonetics, San Diego, CA, USA) containing supplementary growth factors. The cells were cultured at 37℃ in a water-saturated atmosphere of 95% air and 5% CO_2.

2.2. Bacteria culture

Three clinical isolates of *P. gingivalis* (P1, P4, and P8) were obtained from the diseased sites of periodontitis patients. To examine the role of gingipains of *P. gingivalis*, gingipain mutants of *P. gingivalis* ATCC 33277, a kind gift from Dr Koji Nakayama (Nagasaki University, Nagasaki, Japan) [KDP 129 (*kgp*-), KDP 133 (*rgpA*$^-$ *rgpB*$^-$), and KDP 136 (*kgp*$^-$ *rgpA*$^-$ *rgpB*$^-$)], were also used. Three clinical isolates of *P. gingivalis* were anaerobically (10% CO_2, 5% H_2, and 85% N_2) grown in a brain heart infusion broth (BHI; BD Diagnostic Systems, Sparks, MD, USA) supplemented with 5 μg/ml hemin (Sigma–Aldrich Corporation, St Louis, MO, USA) and 5 μg/ml menadione (Sigma). *P. gingivalis* ATCC 33277 and mutant strains were grown in enriched BHI (containing 37g of BHI, 5g of yeast extract, 1g of L-cystein per 1L) supplemented with 5 μg/ml hemin, 5 μg/ml menadione, and antibiotics [20 μg/ml chloramphenicol (Sigma) for KDP 129; 10 μg/ml erythromycin (Sigma) and 0.7 μg/ml tetracycline (Sigma) for

KDP 133; 10 μg/ml erythromycin, 0.7 μg/ml tetracycline, and 20 μg/ml chloramphenicol for KDP 136] as described previously.[32]

2.3. Experimental periodontitis and measurement of alveolar bone loss

The mouse periodontitis experiments were performed and were approved by the Seoul National University Animal Care and Use committee (No. SNU-121106-2). Mice were maintained under specific pathogen-free conditions in the Laboratory Animal Facility at the School of Dentistry, Seoul National University.

Six-week-old male and female BALB/c mice were given sulphamethoxazole/trimethoprim (Samil, Gyeonggi-do, Korea) in deionized water ad libitum for 10 days and then randomly divided into four groups (sham, P1, P4, and P8, n = 10 per group). Four days after antibiotics treatment, mice were orally inoculated 6 times at 2-day intervals with 1 x 10^9 viable *P. gingivalis* P1, P4, and P8 in 100 μl of 2% carboxymethylcellulose (CMC; Tokyo Chemical Industry, Tokyo, Japan), and sham group received PBS in 2% CMC. To observe the infiltration of neutrophils by *P. gingivalis* clinical isolates within the gingival tissues, mice were orally inoculated with 1 x 10^9 viable *P. gingivalis* 3 days before sacrifice. Four weeks after the last inoculation, all mice were euthanized, and the maxillae were removed and the maxillae were defleshed, immersed overnight in 3% hydrogen peroxide and stained with 1%

methylene blue to measure the alveolar bone loss. The distance between the cemento-enamel junction (CEJ) and alveolar bone crest (ABC) at seven maxillary lingual sites per mouse were measured under a dissecting microscope (40×) with SPOT Advanced software (SPOT Imaging Solutions, SterlingHeights, Ml, USA).

2.4. Flow cytometric invasion assay

HOK-16B cells (6 x 10^4 cells/well) were seeded into 24-well plates 1 day before infection. At the 80% confluence, the cells were incubated with viable 5- (and 6-) carboxy-fluorescein diacetate succinimidyl ester (CFSE; Molecular Probe, Carlsbad, CA, USA)-labeled *P. gingivalis* at an multiplicity of infection (MOI) of 1000 in KGM without antibiotics for 4 hours. Infected HOK-16B cells were washed with PBS and detached with trypsin-EDTA. After quenching the fluorescence of the bacteria bound on the surface with 0.4% trypan blue, the cells were analyzed using a FACSCalibur (BD Bioscience, San Diego, CA, USA). To compare the invasion ability of different strains, the invasion index was calculated as follows: [mean fluorescence intensity (MFI) of infected cells-MFI of negative control cells]/MFI of CFSE-labeled bacteria*100.

2.5. Fluorescent immunostaining and confocal microscopy

To examine the effect of *P. gingivalis* on the levels of TJ proteins, the

fluorescent immunostaining of JAM-A and ZO-1 proteins was performed. HOK-16B cells were seeded on 12-mm cover slides (2.5 x 10^5 cells/slide) and cultured for 72 hours. The cells were infected with *P. gingivalis* at an MOI of 1000 for 8 hours. After washing with PBS, the cells were fixed with 4% paraformaldehyde and treated with 50 mM ammonium chloride for 10 min to quench the auto fluorescence. After blocking with 5% BSA, the cells were incubated anti-JAM-A (Abnova, Taipei, Taiwan) or anti-ZO-1 (Invitrogen, Carlsbad, CA, USA) antibody, and then with Alexa 488 anti-rabbit antibody (Invitrogen) or Alexa 555 anti-mouse antibody (Invitrogen) for 1 h. The cells were washed and mounted using ProLongGold anti-fade reagent with DAPI (Invitrogen). For each slide, nine areas were photographed at 100X magnification using a Zeiss LSM700 (Carl Zeiss, Oberkochen, Germany) with serial z-sections, and then the images with maximum intensity projections were obtained by combining the multiple z-sections. The relative fluorescence intensities of TJ proteins were analyzed using ZEN 2010 software (Carl Zeiss) and normalized to the fluorescence intensity of DAPI. Each experiment was performed twice.

2.6. Measurement of transepithelial electrical resistance (TER)

HOK-16B cells (1 x 10^5 cells/well) were seeded on a 3 μm-pore-size polycarbonate filter of a 24-well plate of the transwell two-chamber tissue culture system (SPL Life Sciences, Gyeonggi-do, Korea). The cells were

cultured for 2 or 3 days until a confluent monolayer reached the peak resistance of about 15 Ω. Baseline resistance was measured using ERS Volt-Ohm Meter (Millipore Bedford, MA, USA). After measurement of baseline resistance, the cells were infected with *P. gingivalis* at an MOI of 1000 and then TER was measured at 2, 4, 8, and 16 hours. TER was calculated as a relative percentage of vehicle control at the baseline.

2.7. *In-situ* hybridization

To prepare the *P. gingivalis*-specific probe, a 344-bp DNA fragment of *P. gingivalis*16S rRNA was amplified by PCR using the following primers: 5'-TGC AAC TTG CCT TAC AGA GG-3' and 5'-ACT CGT ATC GCC CGT TAT TC-3'. After precipitation of PCR products, amplified products were labeled with digoxigenin-dUTP by random priming using a DIG DNA labeling and detection kit (Roche Applied Science, Penzberg, Germany) to produce probes. To develop the signal, the premixed substrate solution consisting of nitroblue tetrazolium and 5-bromocresyl-3-indolyphosphate (Roche Applied Science) were distributed onto each specimen and incubated for 25 min. The sections were then counterstained with 0.5% methyl green and mounted. As a negative controls, parallel hybridizations were performed with each probe mixed with a 10-fold excess amount of unlabeled probe. Both buccal and lingual gingival tissue sites were photographed at 400x magnification, and the

signal intensities of bacterial invasion were analyzed using ImageJ software (National Institute of Mental Health, Bethesda, MD, USA). Additional sections were stained with hematoxylin and eosin.

2.8. Immunohistochemistry

Gingival sections were deparaffinized, rehydrated, and incubated with polyclonal anti-Ly6G antibody (Bioss, Woburn, MA, USA) and monoclonal anti-CD3 antibody (abcam, Cambridge, MA, USA). The bound primary antibodies were detected using DAKO Envision™ + Dual Link System-HRP kit (Dako, Santa Barbara, CA, USA). The sections were then counterstained with hematoxylin and mounted. The number of Ly6G- or CD3-positive cells within the mice gingival tissues was counted in the images taken at 200x magnification.

2.9. Measurement of *P. g*-specific IgG2a, IgG1, and IgA antibodies in mice sera

To determine the levels of *P. gingivalis*-specific IgG2a, IgG1, and IgA antibodies, sera were obtained from mouse of *P. gingivalis*-infected or sham group at four weeks after the last inoculation. The levels of antibodies against ethanol-killed *P. gingivalis* were measured using enzyme-linked immunosorbent assay (ELISA). Briefly, *P. gingivalis* P1, P4, and P8 were

treated overnight with 70% ethanol, washed, diluted to an optical density (OD) at 600 nm of 0.1 and coated the wells of 96-well EIA/RIA microtiter plates at 4℃ overnight for each group. For the sham control, mixed *P. gingivalis* antigens were used. After blocking with 2% BSA in PBS for 90 min, the plates were incubated with diluted mice sera (1:25) for 1 hour. After washing, the plates were incubated with HRP-conjugated goat anti-mouse IgG2a (1:5000, SouthernBiotech, Birmingham, AL, USA), IgG1 (1:5000, SouthernBiotech), or IgA (1:8000, SouthernBiotech) antibody at room temperature for 1 hour. Then bound detection Ab was developed with 3, 3', 5, 5'-tetramethylbenzidine substrate (Sigma) for 10 min and the reaction was terminated using 2N H_2SO_4. The absorbance was measured at 450 nm.

2.10. Cytokine measurement

Cervical lymph nodes were obtained from each mouse. To measure the cytokine, cervical lymph nodes were lysed in a 2X cell lysis buffer (Cell Signaling Technology, MA, USA), homogenized, centrifuged for 10 min at 4℃ and the amount of IFN-γ, IL-4, and transforming growth factor (TGF)-β1 in the supernatants was measured using ELISA kit (R&D Systems, MN, USA) according to the manufacturer's instructions.

2.11. Statistical analysis

The differences among all test groups were analyzed using One-way ANOVA with Tukey's post hoc. In the experiments using animal samples, the differences among experimental groups were analyzed using Kruskal-Wallis cltest followed by Mann-Whitney U test with Bonferroni adjustment. The differences between sham and experimental groups were analyzed with Mann-Whitney U test. A *P* value of <0.05 was considered statistically significant. All analyses were performed using SPSS 22.0 (SPSS Inc, IL, USA) and R 3.2.2 software (R Core Team).

3. Results

3.1. Effect of *P. gingivalis* clinical isolates on the epithelial physical barrier function

To clarify the role of invasion ability and proteolytic activity in the virulence of *P. gingivalis*, three strains of *P. gingivalis* clinical isolates (P1: low invasion ability and low proteolytic activity, P4: low invasion ability and high proteolytic activity, P8: high invasion ability and low proteolytic activity) were used. Because the disruption of physical barrier induces periodontitis in mice,[29] the effect of three strains on the physical barrier function of gingival epithelial cells were compared. The measurement of TER has been widely used to determine the integrity of the physical barrier function,[33] and the TER of HOK-16B cells was measured after infection with *P. gingivalis in vitro*. TER decreased in all *P. gingivalis*-infected cells within 4 hours of infection compared to the control cells. After 4 hours of infection, all three strains significantly decreased TER, however, an inter-strain difference was observed between P1 and P4 at 8 hours (Fig. 1A).

The effect of *P. gingivalis* on the levels of TJ proteins was also examined. Infection of P4 markedly reduced the levels of JAM-A protein in HOK-16B cells but P1 and P8 had no effect (Fig. 1B and C). The levels of ZO-1 protein was significantly decreased by all three strains, thus, the levels of both proteins were significantly reduced by P4-infection (Fig. 1B and C). Taken together, the

proteolytic activity of *P. gingivalis* was involved in the disruption of the epithelial physical barrier.

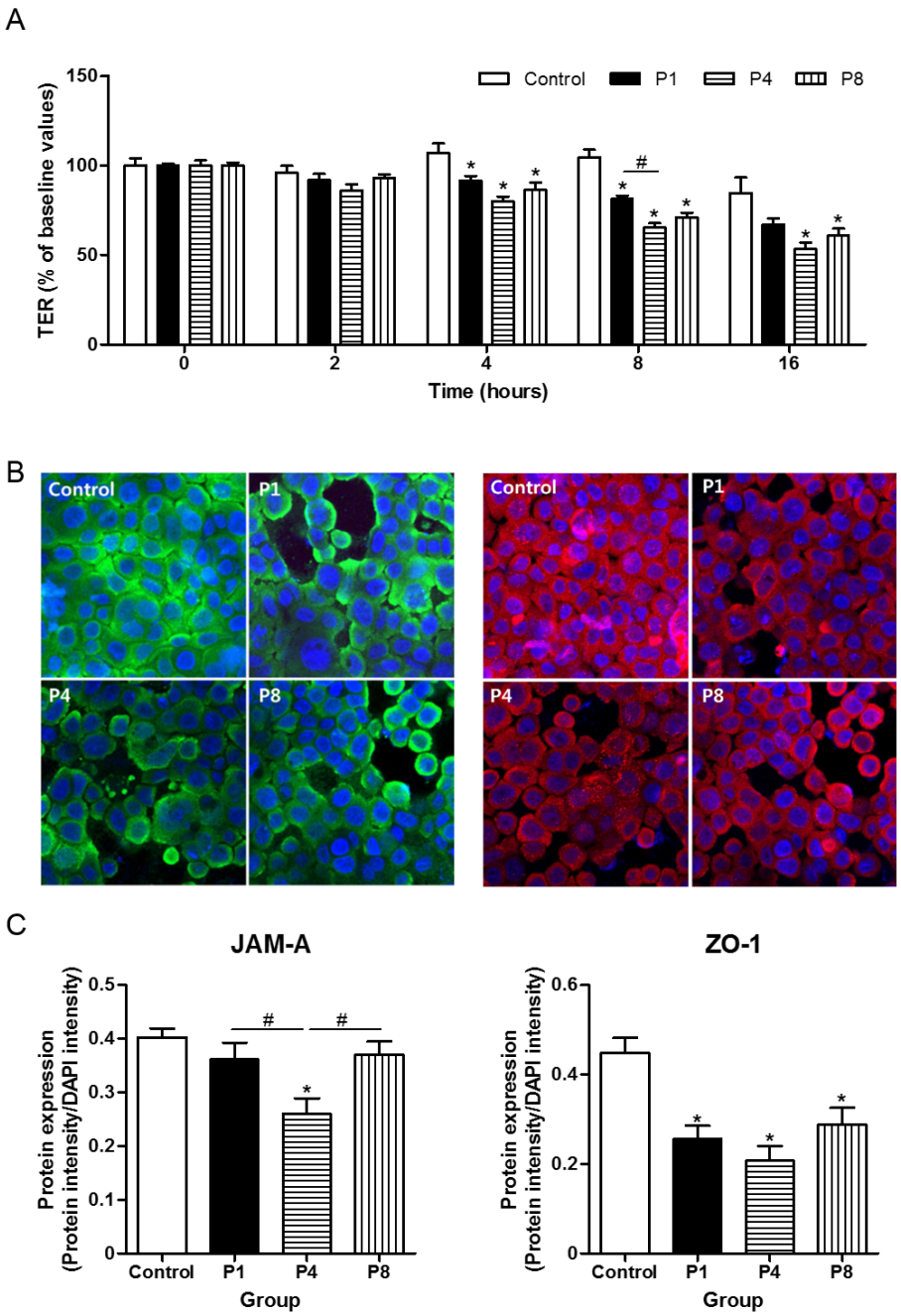

Figure 1. Regulation of epithelial physical barrier by *P. gingivlias*. (A)

Tight-junctioned monolayers of HOK-16B cells were infected with 3 clinical isolates of *P. gingivalis* and TER of monolayers were measured at 2, 4, 8, and 16 hours. Values are given as % baseline. Each value represents the mean ± standard error of the mean. *$p < 0.05$ versus control, #$p < 0.05$ versus P4 strain (One-way ANOVA with Tukey's post hoc). HOK-16B cells grown in monolayers and were incubated with 3 clinical isolates of *P. gingivalis* (P1, P4, and P8) for 8 hours. The cells were fixed and stained for TJ proteins JAM-A and ZO-1 by immunofluorescence and examined by confocal microscopy. (B) Representative images of stained JAM-A (Green), ZO-1 (Red), and nuclei (Blue). (C) The relative fluorescence intensity of stained JAM-A and ZO-1 was analyzed using ZEN 2010 software and normalized to the fluorescence intensity of nuclei. Each column represents the mean ± standard error of 18 data points obtained from two independent experiments. *$p < 0.05$ versus control, #$p < 0.05$ versus P4 strain (One-way ANOVA with Tukey's post hoc).

3.2. The role of gingipain in the bacterial invasion and the modulation of the epithelial physical barrier function

It has been reported that the purified Kgp gingipain is responsible for the degradation of adherens junction protein of Madin-Darby canine kidney (MDCK) epithelial cells.[28] To determine which gingipain play a critical role for the *P. gingivalis* invasion and decreased TER, wild-type *P. gingivalis* ATCC 33277 and isogenic gingipain mutants were used.

The invasion of wild-type *P. gingivalis* and gingipain mutant strains into HOK-16B cells was examined by a flow cytometric invasion assay. The invasion ability of KDP 136 strain was significantly decreased compare with those of wild-type strain, confirming that the gingipain of *P. gingivalis* has an import role in their invasion into host cells. The invasion index of KDP 129 strain tended to be lower than that of KDP 133 strain, indicating that Kgp gingipain has a greater role than Rgp gingipain in the bacterial invasion of epithelial cells (Fig. 2A).

To determine the role of gingipain for decreased TER, the TER was measured using gingipain mutant strains *in vitro*. After 8 hours, wild-type *P. gingivalis* induced a decrease in TER up to 32 hours. At 32 hours after infection, the TER was significantly decreased only by wild-type and KDP 133 strain, suggesting that the epithelial physical barrier may be modulated mainly by Kgp gingipain (Fig 2B).

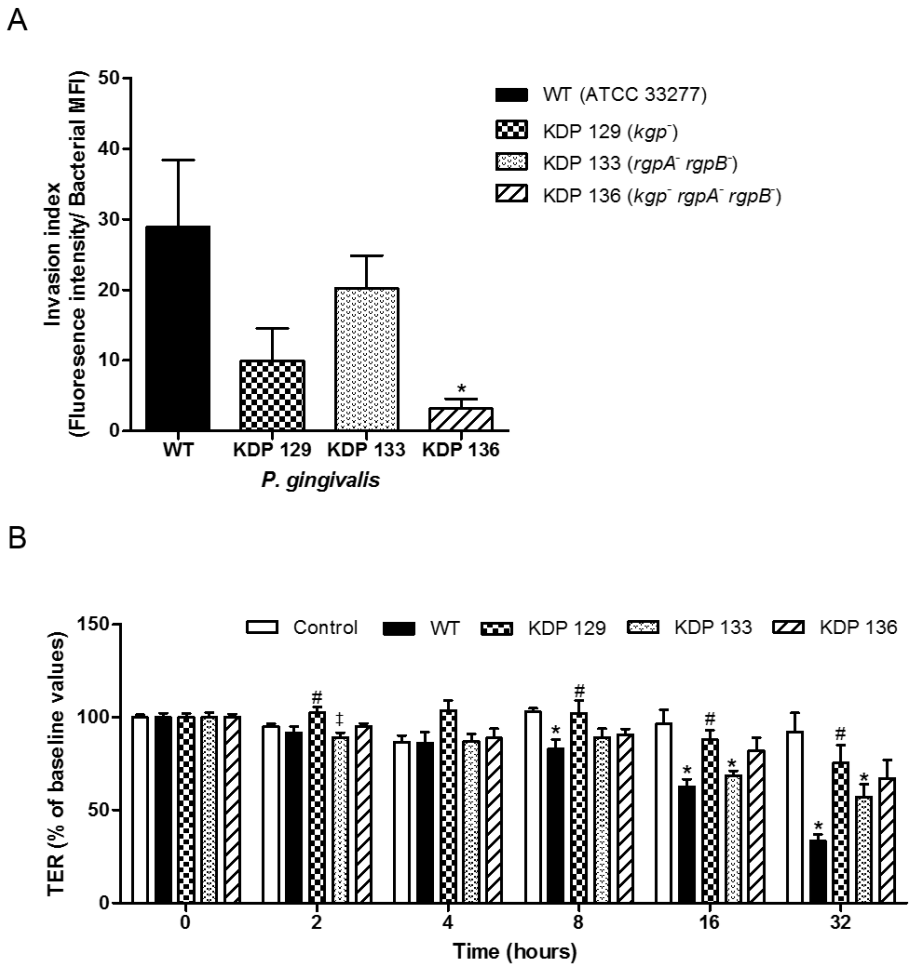

Figure 2. Invasion into HOK-16B cells and regulation of epithelial physical barrier by *P. gingivalis* gingipain mutants. HOK-16B cells (6 x 10^4 cells/well) were seeded into 24-well plates and infected with viable CFSE-labeled *P. gingivalis* at an MOI of 1000 for 4 hours. (A) After quenching the fluorescence of bacteria bound on the cell surface with trypan blue, the fluorescence of HOK-16B cells containing intracellular bacteria was analyzed by flow cytometry. *$p<$

0.05 versus WT strain (One-way ANOVA with Tukey's post hoc). (B) HOK-16B cells were incubated in the absence or presence of *P. gingivalis* at an MOI of 1000. TER was measured at 2, 4, 8, 16, and 32 hours. Values are given as % baseline. *$p < 0.05$ versus control, #$p < 0.05$ versus wild-type strain, ‡$p < 0.05$ versus KDP 129 strain (One-way ANOVA with Tukey's post hoc). Each value represents the mean ± standard error.

3.3. Effect of *P. gingivalis* clinical isolates on alveolar bone loss in the murine model of periodontitis

The effect of *P. gingivalis* clinical isolates with different invasion ability and proteolytic activity on alveolar bone loss was evaluated in mice. After inoculation of *P. gingivalis*, the distances between the cemento-enamel junction and alveolar bone crest at seven maxillary lingual sites were measured (Fig. 3A). P1 with low invasion ability and low proteolytic activity induced alveolar bone loss compared with the sham group, but statistical significance was not achieved. P4 with high proteolytic activity and P8 with high invasion ability induced significant alveolar bone loss compared with the sham group. The alveolar bone loss induced by P4 was significantly higher than that induced by either P1 or P8 (Fig. 3B and C). Taken together, the proteolytic activity of *P. gingivalis* was more important to induce the alveolar bone loss in the murine model of periodontitis.

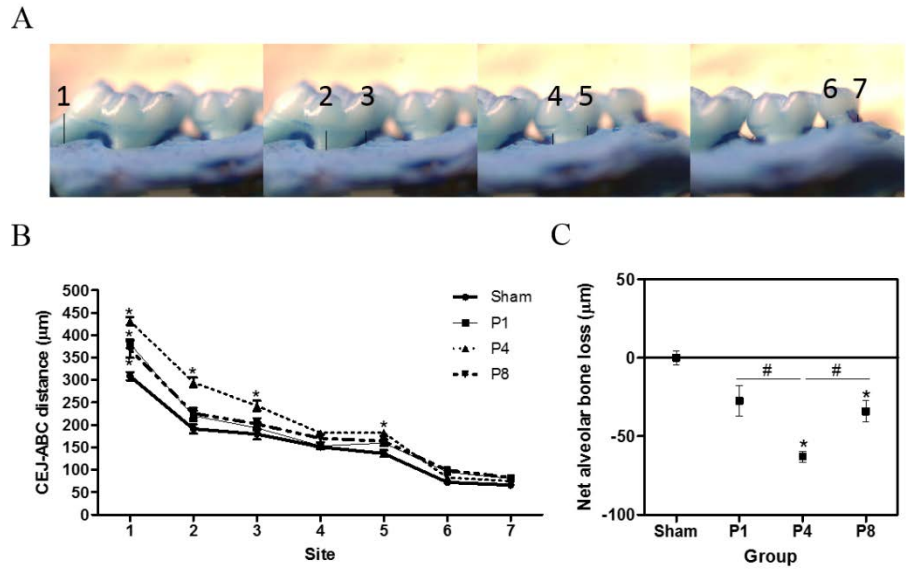

Figure 3. Evaluation of *P. gingivalis*-induced alveolar bone loss. Mice were orally inoculated with 3 clinical isolates of *P. gingivalis* (P1, P4, and P8) or vehicles alone (Sham). (A, B) Distance between the cemento enamel junction and alveolar bone crest (CEJ-ABC) at seven maxillary lingual sites per mouse was measured. **p* < 0.05 versus sham group (Mann-Whitney U test). (C) The alveolar bone loss of each group was calculated. **p* < 0.05 versus sham group, #*p* < 0.05 versus P4 group (Mann-Whitney U test with Bonferroni adjustment).

3.4. The invasion of *P. gingivalis* clinical isolates into the gingival tissues

The disruption of epithelial physical barrier has been related with increased bacteria within the gingival tissues.[29] Therefore, bacteria within the gingival tissues were examined by *in-situ* hybridization using a *P. gingivalis*-specific probe. *P. gingivalis* was detected within the gingival epithelia and lamina propria (Fig. 4A). When the levels of *P. gingivalis* detected within the gingival tissues were measured, the invasion of P4 was increased compare to either P1 or P8 but statistical significance was not achieved (Fig. 4B). In addition, the morphology of bacteria with rod-shape or fusiform was observed in some tissue sections of the sham group. Pyrosequencing data have been shown that the high levels of *Proteus penneri* in the sham group compare with those of *P. gingivalis*-infected group in mice.[29] However, the sequences of *P. gingivalis*-specific probe was not matched with the sequences of *Proteus penneri* 16S rRNA in the database. These results suggest that the proteolytic activity of *P. gingivalis* may play a critical role in process of bacterial invasion into the gingival tissues.

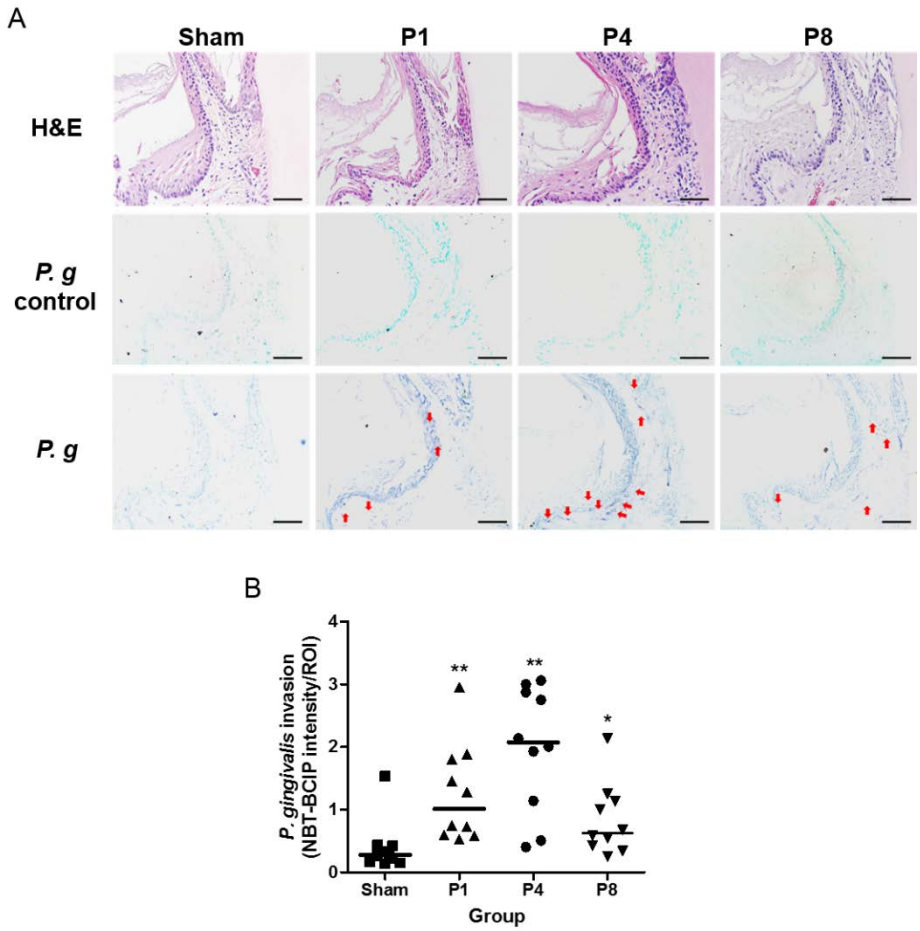

Figure 4. The invasion of *P. gingivalis* within the gingival tissues. (A) Gingival sections from mice were stained with hematoxylin–eosin (H&E) or *in-situ* hybridized with a *P. gingivalis*-specific probe (*P. g*). As a negative control (*P. g* control), hybridization was also performed with probe mixed with 10-fold excess unlabeled probe. Positive signals, shown in violet, are marked with red arrows (scale bar = 50 μm, 400x). (B) The number of detected signals

was analyzed using the ImageJ software. *$p < 0.05$, **$p < 0.01$ versus sham group (Mann-Whitney U test).

3.5. Infiltration of immune cells by *P. gingivalis* clinical isolates within the gingival tissues

Multiple cell types of the innate and adaptive immunity have been detected in gingival tissues from periodontitis patients.[34] Among various cells, neutrophils are the most common leukocyte and are required for periodontal tissue homeostasis.[35] To assess the degree of neutrophil infiltration by *P. gingivalis* inoculation, mice were orally inoculated with viable *P. gingivalis* 3 days before sacrifice and then the number of infiltrated neutrophils was counted by immunohistochemical staining for Ly6G. The neutrophils were normally observed within the lamina propria or between the alveolar bone and the roots (Fig. 5A). Different from expectations, the number of neutrophils was not significantly different among the groups but it was slightly increased by *P. gingivalis* without statistical significance (Fig. 5B).

T cells are recruited to infection sites during inflammatory responses. Increased infiltration of T cells within the gingival tissues from *P. gingivalis*-infected mice has been reported.[29] To determine the infiltration of T cells into the gingival tissues, the number of T cells was assessed by immunohistochemical staining for CD3. T cells were normally observed within the epithelial layer or lamina propria (Fig. 5A). Similar with the pattern of neutrophil infiltration, the number of T cells was not different (Fig. 5B).

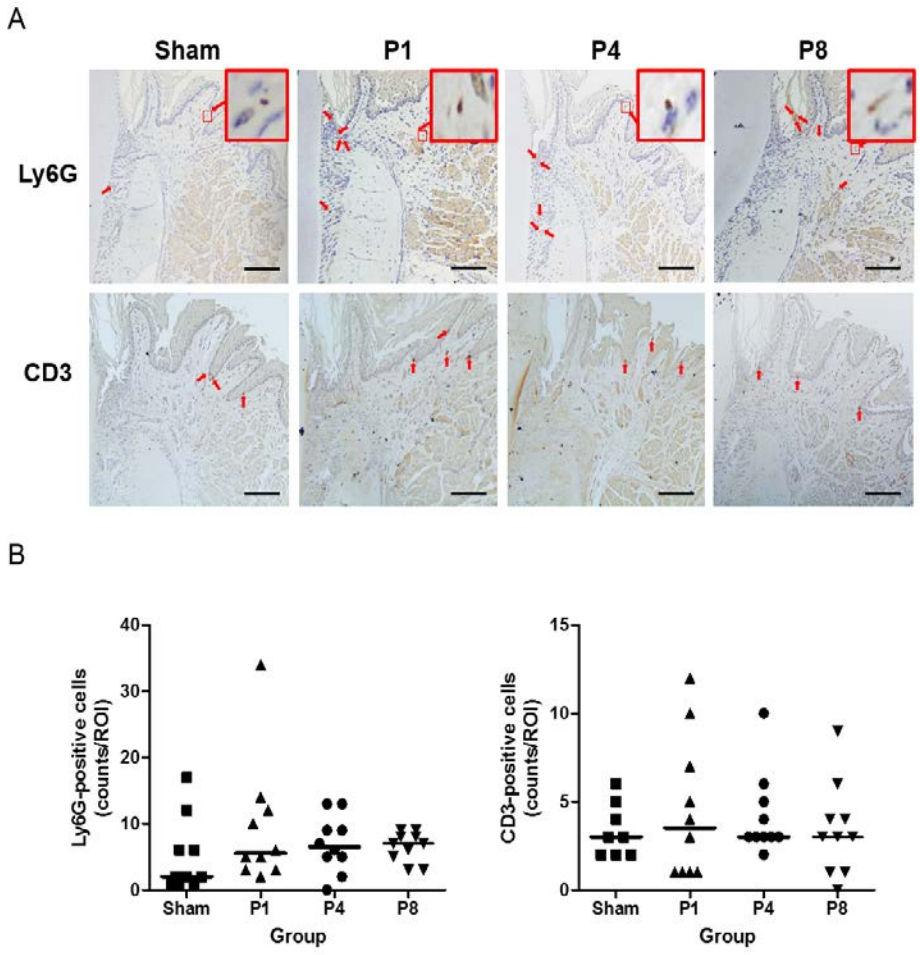

Figure 5. Infiltration of neutrophils and T cells into the gingival tissues after *P. gingivalis* infection. (A) Gingival sections from mice were stained for Ly6G and CD3 by immunohistochemistry. Positive cells are indicated with red arrows (scale bar = 100 μm, 200x). (B) The number of Ly6G- or CD3-positive cells was counted.

3.6. Adaptive immune responses to *P. gingivalis* clinical isolates infection

In general, $CD4^+$ T helper cells are be essential for antibody isotype switching and induction of high-affinity antibody response. Activated T cells secrete various cytokines, which act on the process in heavy chain isotype switching of immunoglobulin.[36,37] Certain cytokines of Th1, Th2, and Th3 cells stimulate the isotype switching to IgG2a, IgG1, and IgA in mice, respectively.[38,39] To determine the effect of *P. gingivalis* with different invasion ability and proteolytic activity on the direction of immune responses, serum IgG2a, IgG1, and IgA titers to *P. gingivalis* were determined by ELISA. The levels of both anti-*P. gingivalis* IgG2a and IgG1 were not increased in all *P. gingivalis*-infected mice compare to the sham group. However, the levels of anti-*P. gingivalis* IgA against P1 were higher than those of the sham group and the levels from P4-infected mice were lower than those of P1- or P8-infected mice (Fig. 6A).

$CD4^+$ cells are distinguished by their cytokine profiles. IFN-γ, IL-4, and TGF-β1 are mainly produced by Th1, Th2, and Th3, respectively.[38,39] To determine the cytokine expression in mice cervical lymph nodes found in the neck, the concentrations of each cytokine was measured by ELISA using lymph node lysates. The levels of these cytokines from all *P. gingivalis*-infected group were significantly decreased compare to the sham group. IFN-γ and IL-4 of P8-infected group were higher than those of P1- or/and P4-infected group. The

levels of TGF-β1 from P4-infected group were lower than those of P1- and P8-infected group (Fig. 6B).

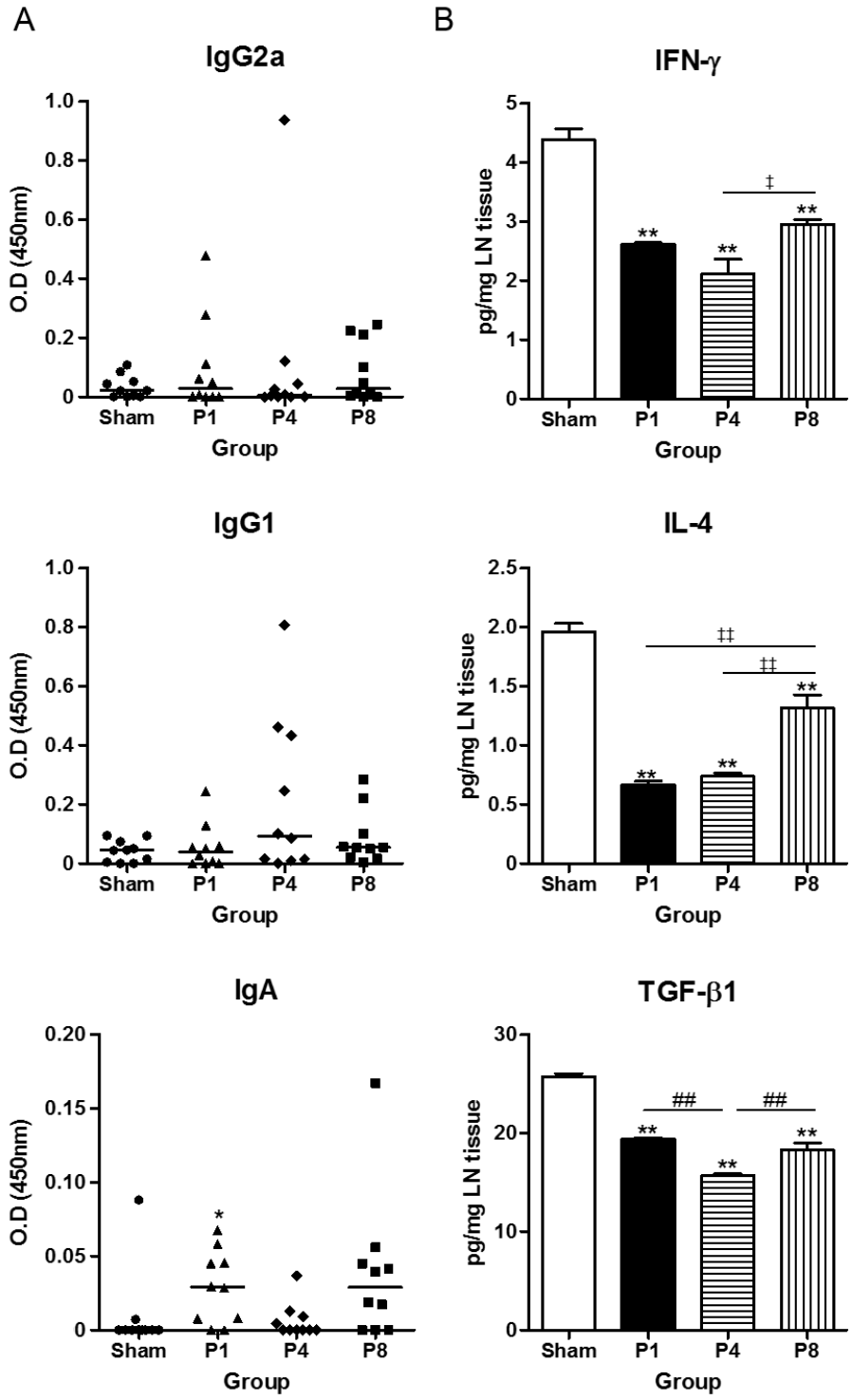
A
IgG2a
O.D (450nm)
1.0
0.8
0.6
0.4
0.2
0
Sham
P1
P4
P8
Group
B
IFN-γ
pg/mg LN tissue
5
4
3
2
1
0
**
‡
IgG1
IL-4
2.5
2.0
1.5
1.0
0.5
‡‡
IgA
0.20
0.15
0.10
0.05
*
TGF-β1
30
20
10
##

Figure 6. The titers of *P. gingivalis*-specific antibodies and the levels of cytokine within cervical lymph node. (A) The levels of *P. gingivalis*-specific antibodies in mice sera were determined by ELISA using ethanol-killed *P. gingivalis* lysates. *$p < 0.05$ versus sham group (Mann-Whitney U test). (B) The concentration of IFN-γ, IL-4, and TGF-β1 in the lysates of mice cervical lymph node were determined by ELISA. **$p<0.01$ versus sham group, ##$p < 0.01$ versus P4 group, ‡$p<0.05$, ‡‡$p<0.01$ versus P8 (One-way ANOVA with Tukey's post hoc).

IV. Discussion

The present study investigated the effect of invasion ability and proteolytic activity of *P. gingivalis* clinical isolates in the murine model of periodontitis in order to understand the underlying mechanism of *P. gingivalis*-induced periodontitis. *P. gingivalis* modulated the epithelial physical barrier through down-regulation of the TJ proteins, JAM-A and ZO-1. Specifically, the Kgp gingipain of *P. gingivalis* was more involved in the bacterial invasion and the impairment of physical barrier *in vitro*. In addition, the induction of alveolar bone loss and the increased bacterial invasion into the gingival tissues in mice were induced primarily by *P. gingivalis* with high proteolytic activity.

Intact epithelium provides a physical barrier to prevent invasion of colonized bacteria. Physical barrier functions are regulated by the expression and/or distribution of TJ proteins. Especially, ZO-1, a cytoplasmic protein, binds to transmembrane proteins (e.g., claudin, occludin, and JAM-A) and links them to cytoskeletal actin. Thus, ZO-1 is a key component in the tight junction structure. In the present study, ZO-1 was more susceptible than JAM-A to degradation by *P. gingivalis*, because ZO-1 levels were also reduced by *P. gingivalis* with relatively low proteolytic activity (P1 and P8), whereas JAM-A levels were induced only by *P. gingivalis* with relatively high proteolytic activity (P4). Although the levels of JAM-A and ZO-1 within the gingival tissues of mice were not measured, the reduction of ZO-1 levels by *P. gingivalis*

in the mice gingival tissues has been reported.[29] It has also been reported that the hemagglutinin domain of Kgp gingipain is responsible for the degradation of the adherens junction protein E-cadherin[28]. Similarly, the decreased TER was induced only by KDP 133, suggesting that the Kgp gingipain may be a key modulator for the regulation of the epithelial physical barrier function.

It has been reported that the Kgp and RgpB gingipain of *P. gingivalis* contribute to the induction of alveolar bone loss in the periodontitis mouse model.[31] In addition, the invasion ability and proteolytic activity of *P. gingivalis* have been related to heterogenic virulence in the mouse abscess model.[30] These findings might help to explain why the P1 strain did not induce significant alveolar bone loss. The greater alveolar bone loss induced by the P4 strain compared to other strains suggests that the proteolytic activity rather than the invasion ability may plays a crucial role in the pathogenesis of the periodontitis murine model. However, it does not coincide with observations regarding humans, where the invasion ability of *P. gingivalis*, but not its cytokine proteolytic activities, exhibited a strong positive correlation with clinical parameters, including mean marginal bone loss.[6] This discrepancy may be attributed to the fact that the junctional and sulcular epithelium are keratinized in mice, thereby preventing the direct invasion of *P. gingivalis* into the epithelial cells.

The increased bacterial number and the persistence of bacteria within the gingival tissues lead to chronic inflammation. *P. gingivalis* ATCC 49417 has

been shown to allow the commensal bacteria to invade the gingival tissues.[29] The number of *P. gingivalis* detected within the gingival tissues appeared to increase particularly in response to inoculation with the P4 strain. This observation might provide an explanation for the increased alveolar bone loss.

Studies have also examined the relationship between periodontitis and defects in the number or function of neutrophils.[35,40,41] However, the present study revealed that the number of infiltrated immune cells (neutrophils and T cells) detected within the gingival tissues was not associated with the induction of alveolar bone loss. Infiltration of neutrophils peaks at 24 hours in gingival tissues after ligature placement in mice has been reported.[42] In *P. gingivalis*-infected mice, neutrophil infiltration increases at 24 hours after inoculation.[43] One possible explanation for this result is that the time of *P. gingivalis* inoculation was not sufficient to observe the infiltration of neutrophils. In addition, the down-regulation of IL-8 as a neutrophil-recruiting chemokine by *P. gingivalis* is well known[44], and decreased expression of CXCL1 (mouse ortholog of human IL-8) at 4 days after *P. gingivalis* inoculation in mice has been reported.[45] Thus, *P. gingivalis* may have the capacity to inhibit or delay neutrophil recruitment into the gingival tissues. Further studies on the identification of other infiltrated immune cells in gingival tissues are needed.

The antibody response to bacteria or its components during chronic inflammation has been shown to have a protective effect. Pro-inflammatory cytokine IFN-γ enhances isotype switching to IgG2a, while anti-inflammatory

cytokine IL-4 enhances IgG1 in mice.[38,39] In the present study, the levels of IgG1 against P4 were slightly increased while the levels of IgA were weakly increased by the P1 and P8 strains. Multiple studies have reported that the antibody response to periodontal bacteria,[46,47] however, the relationship between specific antibody responses against *P. gingivalis* and periodontal diseases is not clear. High IgG antibody levels against *P. gingivalis* in periodontitis patients have been reported.[48,49] In addition, a study revealed that the levels of the anti-*P. gingivalis* antibody are not elevated in the gingival crevicular fluid of periodontitis patients. [50] In the present study, the immune response in a certain direction of T cell response by *P. gingivalis* clinical isolates was not observed. It has been reported that the humoral immune response to *P. gingivalis* in mice is strain-dependent.[51] This finding may help explain why anti-*P. gingivalis* IgG levels did not vary among the experimental groups.

In conclusion, the proteolytic activity, especially involving Kgp gingipain, of *P. gingivalis* was involved in the regulation of epithelial physical barriers through the modulation of tight junction-associated proteins. In addition, the induction of alveolar bone loss and the increased number of invasion sites by *P. gingivalis* with high proteolytic activity provides evidence that the proteolytic activity contributes to pathogenesis in the mice periodontitis model.

V. References

1 Lamont, R. J. & Hajishengallis, G. Polymicrobial synergy and dysbiosis in inflammatory disease. *Trends Mol Med* **21**, 172-183 (2015).

2 Socransky, S. S., Haffajee, A. D., Cugini, M. A., Smith, C. & Kent, R. L., Jr. Microbial complexes in subgingival plaque. *J Clin Periodontol* **25**, 134-144 (1998).

3 Holt, S. C., Kesavalu, L., Walker, S. & Genco, C. A. Virulence factors of *Porphyromonas gingivalis*. *Periodontol 2000* **20**, 168-238 (1999).

4 Imamura, T. The role of gingipains in the pathogenesis of periodontal disease. *J Periodontol* **74**, 111-118 (2003).

5 Potempa, J., Sroka, A., Imamura, T. & Travis, J. Gingipains, the major cysteine proteinases and virulence factors of *Porphyromonas gingivalis*: structure, function and assembly of multidomain protein complexes. *Curr Protein Pept Sci* **4**, 397-407 (2003).

6 Baek, K. J., Ji, S., Kim, Y. C. & Choi, Y. Association of the invasion ability of *Porphyromonas gingivalis* with the severity of periodontitis. *Virulence* **6**, 274-281 (2015).

7 Calkins, C. C., Platt, K., Potempa, J. & Travis, J. Inactivation of tumor necrosis factor-alpha by proteinases (gingipains) from the periodontal pathogen, *Porphyromonas gingivalis*. Implications of immune evasion. *J Biol Chem* **273**, 6611-6614 (1998).

8 Mikolajczyk-Pawlinska, J., Travis, J. & Potempa, J. Modulation of interleukin-8 activity by gingipains from *Porphyromonas gingivalis*: implications for pathogenicity of periodontal disease. *FEBS Lett* **440**, 282-286 (1998).

9 Stathopoulou, P. G., Benakanakere, M. R., Galicia, J. C. & Kinane, D. F. The host cytokine response to *Porphyromonas gingivalis* is modified by gingipains. *Oral Microbiol Immunol* **24**, 11-17 (2009).

10 Tam, V. *et al.* The RgpA-Kgp proteinase-adhesin complexes of *Porphyromonas gingivalis* Inactivate the Th2 cytokines interleukin-4 and interleukin-5. *Infect Immun* **77**, 1451-1458 (2009).

11 Yun, L. W., Decarlo, A. A., Collyer, C. & Hunter, N. Enhancement of Th2 pathways and direct activation of B cells by the gingipains of *Porphyromonas gingivalis*. *Clin Exp Immunol* **134**, 295-302 (2003).

12 Hornef, M. W., Wick, M. J., Rhen, M. & Normark, S. Bacterial strategies for overcoming host innate and adaptive immune responses. *Nat Immunol* **3**, 1033-1040 (2002).

13 Finlay, B. B. & McFadden, G. Anti-immunology: evasion of the host immune system by bacterial and viral pathogens. *Cell* **124**, 767-782 (2006).

14 Lamont, R. J. *et al.* *Porphyromonas gingivalis* invasion of gingival epithelial cells. *Infect Immun* **63**, 3878-3885 (1995).

15 Deshpande, R. G., Khan, M. B. & Genco, C. A. Invasion of aortic and heart endothelial cells by *Porphyromonas gingivalis*. *Infect Immun* **66**, 5337-5343 (1998).

16 Dorn, B. R., Burks, J. N., Seifert, K. N. & Progulske-Fox, A. Invasion of endothelial and epithelial cells by strains of *Porphyromonas gingivalis*. *FEMS Microbiol Lett* **187**, 139-144 (2000).

17 Amornchat, C., Rassameemasmaung, S., Sripairojthikoon, W. & Swasdison, S. Invasion of *Porphyromonas gingivalis* into human gingival fibroblasts in vitro. *J Int Acad Periodontol* **5**, 98-105 (2003).

18 Nakagawa, I. *et al.* Invasion of epithelial cells and proteolysis of cellular focal adhesion components by distinct types of *Porphyromonas gingivalis* fimbriae. *Infect Immun* **74**, 3773-3782 (2006).

19 Njoroge, T., Genco, R. J., Sojar, H. T., Hamada, N. & Genco, C. A. A role for fimbriae in *Porphyromonas gingivalis* invasion of oral epithelial cells. *Infection and Immunity* **65**, 1980-1984 (1997).

20 Chen, T. & Duncan, M. J. Gingipain adhesin domains mediate *Porphyromonas gingivalis* adherence to epithelial cells. *Microb Pathog* **36**, 205-209 (2004).

21 Kato, T. *et al.* Maturation of fimbria precursor protein by exogenous gingipains in *Porphyromonas gingivalis* gingipain-null mutant. *FEMS Microbiol Lett* **273**, 96-102 (2007).

22 Choi, Y. S., Kim, Y. C., Baek, K. J. & Choi, Y. In Situ Detection of Bacteria within Paraffin-embedded Tissues Using a Digoxin-labeled DNA Probe Targeting 16S rRNA. *Jove-J Vis Exp*, doi:ARTN e5283610.3791/52836 (2015).

23 Ji, S., Choi, Y. S. & Choi, Y. Bacterial invasion and persistence: critical events in the pathogenesis of periodontitis? *J Periodontal Res* **50**, 570-585 (2015).

24 Gonzalez-Mariscal, L., Betanzos, A., Nava, P. & Jaramillo, B. E. Tight junction proteins. *Prog Biophys Mol Biol* **81**, 1-44 (2003).

25 Rajasekaran, S. A., Beyenbach, K. W. & Rajasekaran, A. K. Interactions of tight junctions with membrane channels and transporters. *Bba-Biomembranes* **1778**, 757-769 (2008).

26 Kimura, K. *et al.* Protection of Human Corneal Epithelial Cells From TNF-alpha-Induced Disruption of Barrier Function by Rebamipide. *Invest Ophth Vis Sci* **54**, 2752-2760 (2013).

27 Katz, J., Sambandam, V., Wu, J. H., Michalek, S. M. & Balkovetz, D. F. Characterization of *Porphyromonas gingivalis*-induced degradation of epithelial cell junctional complexes. *Infect Immun* **68**, 1441-1449 (2000).

28 Katz, J. *et al.* Hydrolysis of epithelial junctional proteins by *Porphyromonas gingivalis* gingipains. *Infect Immun* **70**, 2512-2518 (2002).

29 Choi, Y. S. *et al. Porphyromonas gingivalis* and Dextran Sulfate Sodium Induce Periodontitis through the Disruption of Physical Barriers in Mice. *Eur J Inflamm* **11**, 419-431 (2013).

30 Inaba, H. *et al.* Heterogenic virulence and related factors among clinical isolates of *Porphyromonas gingivalis* with type II fimbriae. *Oral Microbiol Immunol* **23**, 29-35 (2008).

31 Pathirana, R. D., O'Brien-Simpson, N. M., Brammar, G. C., Slakeski, N. & Reynolds, E. C. Kgp and RgpB, but not RgpA, are important for *Porphyromonas gingivalis* virulence in the murine periodontitis model. *Infect Immun* **75** (2007).

32 Shi, Y. *et al.* Genetic analyses of proteolysis, hemoglobin binding, and hemagglutination of *Porphyromonas gingivalis*. Construction of

mutants with a combination of rgpA, rgpB, kgp, and hagA. *J Biol Chem* **274**, 17955-17960 (1999).

33 Benson, K., Cramer, S. & Galla, H. J. Impedance-based cell monitoring: barrier properties and beyond. *Fluids Barriers CNS* **10**, 5, doi:10.1186/2045-8118-10-5 (2013).

34 Kim, Y. C. *et al.* Presence of *Porphyromonas gingivalis* and plasma cell dominance in gingival tissues with periodontitis. *Oral Dis* **16**, 375-381 (2010).

35 Scott, D. A. & Krauss, J. Neutrophils in periodontal inflammation. *Front Oral Biol* **15**, 56-83 (2012).

36 Sha, Z. & Compans, R. W. Induction of CD4(+) T-cell-independent immunoglobulin responses by inactivated influenza virus. *J Virol* **74**, 4999-5005 (2000).

37 King, C. New insights into the differentiation and function of T follicular helper cells. *Nat Rev Immunol* **9**, 757-766 (2009).

38 Jegerlehner, A. *et al.* TLR9 signaling in B cells determines class switch recombination to IgG2a. *J Immunol* **178**, 2415-2420 (2007).

39 Min, K. M. & Kim, P. H. Macrophage-derived TGF-beta 1 induces IgA isotype expression. *Mol Cells* **16**, 245-250 (2003).

40 Roberts, H. M. *et al.* Impaired neutrophil directional chemotactic accuracy in chronic periodontitis patients. *J Clin Periodontol* **42**, 1-11 (2015).

41 Hajishengallis, G., Chavakis, T., Hajishengallis, E. & Lambris, J. D. Neutrophil homeostasis and inflammation: novel paradigms from studying periodontitis. *J Leukoc Biol* **98**, 539-548 (2015).

42 Shin, J. *et al.* DEL-1 restrains osteoclastogenesis and inhibits inflammatory bone loss in nonhuman primates. *Sci Transl Med* **7**, 307ra155 (2015).

43 Yu, J. J. *et al.* An essential role for IL-17 in preventing pathogen-initiated bone destruction: recruitment of neutrophils to inflamed bone requires IL-17 receptor-dependent signals. *Blood* **109**, 3794-3802 (2007).

44 Darveau, R. P., Belton, C. M., Reife, R. A. & Lamont, R. J. Local chemokine paralysis, a novel pathogenic mechanism for P*orphyromonas gingivalis*. *Infection and Immunity* **66**, 1660-1665 (1998).

45 Hajishengallis, G. *et al.* Low-abundance biofilm species orchestrates inflammatory periodontal disease through the commensal microbiota and complement. *Cell Host Microbe* **10**, 497-506 (2011).

46 Nakagawa, S. *et al.* Infection by *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*, and antibody responses at different ages in humans. *J Periodontal Res* **29**, 9-16 (1994).

47 Gemmell, E. *et al.* Modulation of the antibody response by *Porphyromonas gingivalis* and *Fusobacterium nucleatum* in a mouse model. *Oral Microbiol Immunol* **19**, 247-251 (2004).

48 Yusof, Z., Porter, S. R., Greenman, J. & Scully, C. Levels of serum IgG against *Porphyromonas gingivalis* in patients with rapidly progressive periodontitis, rheumatoid arthritis and adult periodontitis. *J Nihon Univ Sch Dent* **37**, 197-200 (1995).

49 Franca, M. *et al.* Humoral immune response to antigens of *Porphyromonas gingivalis* ATCC 33277 in chronic periodontitis. *J Appl Oral Sci* **15**, 213-219 (2007).

50 Baranowska, H. I., Palmer, R. M. & Wilson, R. F. A comparison of antibody levels to Bacteroides gingivalis in serum and crevicular fluid from patients with untreated periodontitis. *Oral Microbiol Immunol* **4**, 173-175 (1989).

51 Wilensky, A. *et al.* Strain-dependent activation of the mouse immune response is correlated with *Porphyromonas gingivalis*-induced experimental periodontitis. *J Clin Periodontol* **36**, 915-921 (2009).

국문초록

치주염 생쥐모델에서 *Porphyromonas gingivalis*의 침투 능력과 단백질 분해 능력의 역할

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(지도교수: 최 영 님)

1. 목 적

치주염은 *Porphyromonas gingivalis*를 포함하는 다양한 구강 세균의 감염에 의한 만성 염증성 질환이다. 주요 병인 세균으로 알려져 있는

*P. gingivalis*는 그람 음성 혐기성 세균이며, 단백질 분해효소 (gingipains), LPS, capsule, fimbriae와 같은 다양한 병독력 인자를 가지고 있다. 그 중 단백질 분해효소인 gingipain은 사이토카인 신호 전달 체계를 방해하여 숙주의 면역방어를 회피하기 위한 주요 병독력 인자 중 하나로 알려져 있으며, 사이토카인/케모카인, 보체 단백질을 포함하는 숙주의 다양한 단백질을 분해할 수 있는 것으로 알려져 있다. 또한 *P. gingivalis*는 치은 섬유아세포, 치은 상피세포, 혈관 내피세포 등 다양한 숙주 세포 내로 침투할 수 있는 능력을 가지고 있다. 이러한 단백질 분해 능력과 세포 내 침투 능력은 균주마다 차이가 있는 것으로 확인되었다.

구강의 상피세포는 화학적, 물리적, 면역학적 기능을 통해 숙주 내로 침투하는 세균에 대항하기 위한 방어막 역할을 한다. 특히 물리적 장벽은 junctional adhesion molecules (JAMs), zonula occludens (ZOs)과 같은 밀착연접 단백질의 발현에 의해 조절이 되는데, *P. gingivalis*의 gingipain은 상피 접합 단백질의 발현을 조절할 수 있는 것으로 보고되었다. 또한 상피의 물리적 장벽의 파괴는 치주염의 병인 기전과도 연관되어 있다고 알려져 있다.

서로 다른 침투 능력과 단백질 분해 능력을 가진 *P. gingivalis*가 치주염의 병인 기전에서 어떠한 역할을 하는지 동시에 비교한 연구는 진행된 적이 없기 때문에, *P. gingivalis*의 2가지 능력이 치주염의 발병에 미치는 영향과 면역 반응을 확인해보고자 생쥐 모델을 이용하여 평가하

였다.

2. 방 법

치주염 환자로부터 분리한 임상 균주 (P1, P4, P8)를 실험에 이용하였다. P1 균주는 침투 능력과 단백질 분해 능력이 낮으며, P4는 침투 능력이 낮지만 단백질 분해 능력은 높다. 그리고 P8 균주는 침투 능력은 높지만 단백질 분해 능력은 낮다.

*P. gingivalis*가 상피세포의 물리적 장벽에 미치는 영향을 확인하기 위해서 치은 상피세포 (HOK-16B)를 배양하여 단층을 형성한 후에, transepithelial electrical resistance (TER)를 측정하고, 밀착연접 관련 단백질인 JAM-A와 ZO-1의 발현을 형광 면역조직화학법으로 확인하였다.

*P. gingivalis*의 세포 내 침투와 상피의 물리적 장벽 조절에 gingipain이 어떤 역할을 하는지 확인해보고자, *P. gingivalis* ATCC 33277 (WT), gingipain 변이주 [KDP 129 (*kgp*-), KDP 133 (*rgpA*$^{-}$*rgpB*$^{-}$), KDP 136 (*kgp*$^{-}$*rgpA*$^{-}$*rgpB*$^{-}$)]를 이용하였다. 치은 상피세포를 CFSE로 염색한 *P. gingivalis*로 감염시킨 후 유세포 분석기를 이용하여 침투 능력을 확인하였으며, TER을 측정하여 상피의 물리적 장벽 기능을 확인하였다.

치주염의 병인 기전에서 *P. gingivalis*의 세포 내 침투 능력과 단백질 분해 능력의 역할을 확인하기 위하여, 임상 균주인 P1, P4, P8을 각각

생쥐 (n=10)의 구강 내로 6회 접종하여 (1×10^9/마리 당) 치주염을 유도 하였으며 4주 후 희생하기 3 일전 1 회 더 접종하였다. 희생한 생쥐의 치조골 손실을 정량 하였고, 치은 조직 내로 침투한 *P. gingivalis*를 확인하기 위하여, *P. gingivalis*-특이적 프로브를 제작하여 *in-situ* hybridization 방법을 통해 염색하여 정량 하였다. 또한 치은 조직 내에서 호중구 (neutrophil)와 T세포의 존재를 확인하기 위하여, 각 세포의 표지자에 대한 항체를 이용하여 면역조직화학법을 시행하였다. *P. gingivalis* 임상 균주에 대한 적응 면역반응의 방향을 확인해보고자, 생쥐 혈청 내에 존재하는 *P. gingivalis*-특이적 IgG2a, IgG1, IgA 항체의 양과 경부 임파선 (cervical lymph node) 내의 IFN-γ, IL-4, TGF-β1의 양을 효소 면역측정법으로 정량 하였다.

3. 결 과

모든 *P. gingivalis* 임상 균주에 의해 TER이 유의하게 감소하였으며, 특히 8시간 후 P1과 P4 균주 사이에서 유의한 차이를 확인 할 수 있었다. 또한 모든 임상 균주에 의해 HOK-16 세포 내에서 밀착연접 단백질인 JAM-A와 ZO-1의 발현이 감소하였고, P4 균주의 효과가 가장 크게 나타났다. Gingipain 변이주 KDP 136 (*kgp-rgpA*$^-$*rgpB*$^-$) 균주는 야생형 균주 (ATCC 33277)와 비교했을 때, 세포 내 침투 능력이 현저하게 감소된 것을 확인하였다. 야생형 균주와 KDP 133 균주만 통계적으로 유의하게 TER을 감소시켰다.

*P. gingivalis*에 의해 유도된 치주염 생쥐 모델에서 치조골 손실을 측정하였더니, P4와 P8 균주에 의해서만 유의하게 치조골 손실이 일어났으며, 특히 P4 균주에 의해 가장 큰 치조골 손실이 야기되었다. *P. gingivalis* 감염 후, 생쥐 치은 조직 내에서 *P. gingivalis*의 침투를 *in-situ* hybridization으로 정량 해보았더니, P4 균주를 감염시킨 생쥐 그룹에서 가장 높은 세균의 침투를 확인할 수 있었다. 그리고 치은 조직 내에서 호중구와 T 세포의 침윤을 비교해보고자, 각 세포의 특이적 표지자인 Ly6G와 CD3에 대한 항체를 이용하여 면역조직화학법으로 확인 한 결과, 그룹 간에 큰 차이를 발견하지는 못하였다. 생쥐 혈청 내에 존재하는 *P. gingivalis*-특이적 IgG2a, IgG1, IgA 항체의 양을 효소면역측정법으로 정량 했을 때, IgG2a와 IgG1은 그룹 간에 차이를 보이지 않았다. 하지만 IgA 항체의 양은 대조군 그룹과 비교했을 때 P1 균주 감염에 의해 통계적으로 유의하게 증가하였다. 마지막으로 경부 임파선 (cervical lymph node) 내의 IFN-γ, IL-4, TGF-β1의 양을 효소 면역측정법으로 정량 한 결과, 3가지 사이토카인의 양을 대조군과 비교 했을 때, 모두 통계적으로 유의하게 감소된 것을 관찰하였다.

4. 결 론

이상의 결과들은 *P. gingivalis*의 단백질 분해 능력이 밀착연접 단백질의 발현을 감소시키고, 상피세포의 물리적 장벽 기능을 조절 할 수 있다는 것을 암시한다. 또한 단백질 분해 능력이 높은 P4 균주에 의해 가장

큰 치조골의 손실이 일어났고, 치은 조직 내에서 더 많은 *P. gingivalis*의 존재를 확인하였기 때문에 생쥐 치주염 모델의 병인 기전에서 *P. gingivalis*의 단백질 분해 능력이 좀 더 중요한 역할을 할 것이라는 것을 예상할 수 있다.

주 요 어: 세포 침투 능력, 단백질 분해 능력, 치주염 병인기전, *Porphyromonas gingivalis*

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