

Porphyromonas gulae 41-kDa fimbriae induced osteoclast differentiation and cytokine production

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ABSTRACT. *Porphyromonas gulae* is considered to be associated with canine periodontitis. We have previously reported that the *P. gulae* American Type Culture Collection (ATCC) 51700 comprised 41-kDa fimbriae. The purpose of the present study was to demonstrate the roles of 41-kDa fimbrial protein in periodontal disease. In this study, we examined the involvement of the 41-kDa fimbrial protein in osteoclast differentiation and cytokine production in murine macrophages. Furthermore, alveolar bone resorption induced by *P. gulae* infection in rats was evaluated. To estimate osteoclast differentiation, bone marrow cells and MC3T3-G2/PA6 cells were cultured with or without the 41-kDa fimbrial protein for 7 days. BALB/c mouse peritoneal macrophages were stimulated with the 41-kDa fimbrial protein, and the levels of interleukin (IL)-1 β and tumor necrosis factor (TNF)- α production were determined by enzyme-linked immunosorbent assay. Osteoclast differentiation was significantly enhanced by treatment with the 41-kDa fimbrial protein in a dose-dependent manner. The total area of pits formed on the dentine slices with osteoclasts incubated with the 41-kDa fimbrial protein was significantly greater than that of the control. The purified 41-kDa fimbrial protein induced IL-1 β and TNF- α production in BALB/c mouse peritoneal macrophages after 6 hr of incubation in a dose-dependent manner. The bone loss level in rats infected with *P. gulae* was significantly higher than that of the sham-infected rats. These results suggest that *P. gulae* 41-kDa fimbriae play important roles in the pathogenesis of periodontal disease.

KEY WORDS: cytokine production, fimbria, osteoclast differentiation, periodontitis, *Porphyromonas gulae*

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Porphyromonas gulae (*P. gulae*) is a black-pigmented, asaccharolytic, anaerobic, nonmotile, non-spore-forming, Gram-negative, rod-shaped organism. In 2001, *P. gulae* was designated as a novel species inhabiting in the gingival sulcus of various animals [4]. DNA–DNA homology data and 16S ribosomal RNA (rRNA) gene sequence analysis provide strong evidence that strains from the animal biotype of *Porphyromonas gingivalis* (*P. gingivalis*) represent a *P. gulae* that is distinct from *P. gingivalis* [4]. This bacterium is encountered significantly more often and in higher quantities in the diseased periodontal pockets than in the healthy gingival sulcus of dogs [29].

Therefore, *P. gulae* is considered to be associated with canine periodontitis [12].

The etiologic factors of gingivitis and periodontitis seem to be identical in humans and dogs [12, 34]. It has been estimated that approximately 80% of dogs and cats demonstrate some degree of periodontal disease by 4 years of age [13, 27]. It is reported that the severity of periodontal disease worsens with age. By age 5, the percentage of *Porphyromonas* spp. isolated from canine plaque is roughly 6-fold higher than that at 2 years of age, and it correlates with disease severity [1].

Hamada *et al.* [8] reported that the 41-kDa protein of *P.*

gulae was a fimbria of this bacterium. The *P. gulae* 41-kDa fimbrial subunit protein (FimA) encoded by *fimA* is thought to correlate with periodontitis [25].

P. gingivalis is a pathogen causing periodontal disease, which is a typical chronic inflammatory disease [5, 20, 22, 36, 37]. The bacterial fimbria is an important cell structure that contributes to the adherence and invasion of host cells [3, 21, 26, 35], and it induces inflammatory processes in periodontal tissues through a number of mechanisms [2, 6]. *P. gingivalis* fimbriae are capable of binding specifically to components lining the oral cavity, such as salivary proteins, commensal bacteria, several types of extracellular matrices and host cells including gingival fibroblasts, epithelial cells and endothelial cells [9]. These adhesive abilities are considered to be a major pathogenic trait that causes periodontal tissue destruction [24]. In addition, Ozaki *et al.* [26] have shown that fimbriae function as virulence factors in inflammatory reactions because they stimulated the production of inflammatory cytokines by macrophages and fibroblasts. These observations suggest the involvement of the fimbriae as regulators of inflammatory reactions due to bacterial infection.

The role of fimbriae of *P. gulae* in periodontitis remains unclear. In this study, we examined the effects of the 41-kDa fimbrial protein from *P. gulae* on the induction of osteoclast differentiation and on inflammatory cytokine production in murine peritoneal macrophages.

MATERIALS AND METHODS

Strains and cultivation conditions: *P. gulae* ATCC 51700 was cultivated in an anaerobic chamber (15% CO₂, 15% H₂

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and 70% N₂) (ANX-1, Hirasawa Works, Tokyo, Japan) at 37°C in pre-reduced brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI, U.S.A.) supplemented with 0.5% yeast extract, 5 µg/ml hemin and 0.2 µg/ml vitamin K₁.

Isolation and purification of the 41-kDa fimbriae from *P. gulae*: *P. gulae* was incubated anaerobically for 18 hr in BHI broth. The bacterial cell pellet was harvested by centrifugation at 8,000 × g for 30 min at 4°C and washed twice with 20 mM Tris-HCl buffer (pH 8.0) containing 10 mM MgCl₂ and 1.5 M NaCl by repeated pipetting. The suspension was subjected to ultrasonication with a 3-mm microtip and an output power of 25 W on the pulse setting with 5 cycles of 1 min in an icebox. The supernatant of the sonic extract was centrifuged at 10,000 × g for 30 min at 4°C and subjected to 40% ammonium sulfate saturation by the stepwise addition of ammonium sulfate. The precipitated protein was collected by centrifugation at 10,000 × g for 30 min at 4°C, suspended in a minimum volume of 20 mM Tris-HCl buffer (pH 8.0) and dialyzed against the same buffer. The dialysate sample containing most of the fimbriae was subjected to further purification on a diethylaminoethyl (DEAE)-Sephacrose CL-6B column (1.5 by 20 cm) equilibrated with 20 mM Tris-HCl buffer (pH 8.0). The column was washed with 20 mM Tris-HCl buffer and then eluted with a linear gradient of 0 M to 0.3 M NaCl. The protein content of the fractions was measured by ultraviolet light adsorption at 280 nm. Lipopolysaccharide (LPS) was not detected on silver-stained gel of the same preparation by a Silver Stain II kit (Wako Pure Chemical Industries, Osaka, Japan). The endotoxicity of the fimbrial protein was not detected by a colorimetric Limulus amoebocyte lysate assay (GenScript, Tokyo, Japan).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE): Protein extracts were heated at 100°C for 5 min in loading buffer. Samples were applied to 12.0% polyacrylamide slab gels with a 4% stacking gel and electrophoresed at with a constant current of 30 mA for 1 hr. The proteins were stained with Coomassie Brilliant Blue R-250. For molecular weight calibration, a low-molecular weight marker kit (ATTO, Tokyo, Japan) was used.

Electron microscopy: The fimbriae of *P. gulae* cells were examined with a JEM-1220 electron microscope (Nippon Denshi Co., Tokyo, Japan). Bacterial cells from an 18-hr anaerobic culture were collected by centrifugation (10,000 × g for 1 min) washed and resuspended (5 × 10⁸/ml) in phosphate-buffered saline (pH 7.4). Ten microliters of the cell suspension or purified fimbriae was applied on a copper grid coated with a thin Formvar film and air-dried. The samples were then negatively stained with 2% (wt./vole) uranyl acetate for 1 min, air-dried, examined and photographed with a transmission electron microscope operating at 80 kV.

Osteoclast differentiation: MC3T3-G2/PA6 (PA6) cells were used in this assay. PA6 cells were established from a newborn C57BL/6N mouse calvaria, which was kindly supplied by Udagawa *et al.* [32]. PA6 cells (1 × 10⁶ cells/well), which function similar to stromal cells derived from bone marrow and bone marrow cells from BALB/c mouse (1 × 10⁷ cells/well), were co-cultured in α -Minimum Essential Media (α -MEM) containing 20% fetal bovine serum (FBS;

Irvine Scientific, Santa Ana, CA, U.S.A.), 10⁻⁸ M dexamethasone (DEX; Sigma, St. Louis, MO, U.S.A.), RANKL (Sigma) and 10⁻⁸ M 1 α ,25(OH)₂D₃ (calcitriol; Wako) with or without of *P. gulae* 41-kDa fimbrial protein and *E. coli* LPS F583 (Sigma). The cells were incubated in 48-well plates (Sumitomo Bakelite, Tokyo, Japan) at 37°C in 5% CO₂ for 7 days. After 7 days, the co-cultured cells were stained with tartrate-resistant acid phosphatase (TRAP), a marker of the enzyme of osteoclasts. TRAP staining solution consisted of Naphthol AS-MX phosphate (Sigma), sodium tartrate (Sigma) and Fast Red Violet LB Salt (Sigma). TRAP-positive multinucleated cells containing three or more nuclei were counted as osteoclasts. The cells without of *P. gulae* 41-kDa fimbrial protein and *E. coli* LPS were used as a negative control and a positive control, respectively.

Pit formation: Pit formation was assayed with the method of Kawata *et al.* [18] with some modifications. The dentine slices (6 mm in diameter, 0.3 mm thick) were prepared with an ethanol-cooled diamond saw (Isomet 2000; Buehler, Lake Bluff, IL, U.S.A.). Osteoclast precursors were prepared as follows. In brief, BALB/c mouse bone marrow cells were co-cultured with MC3T3-G2/PA6 in α -MEM containing 2% type I collagen (Nitta Gelatin, Osaka, Japan), 10% FBS, macrophage colony-stimulating factor, RANKL, DEX and calcitriol for 7 days in 100-mm diameter dishes (Corning Inc., Corning, NY, U.S.A.). Mouse osteoclast precursors were cultured with RANKL, DEX, calcitriol and *P. gulae* fimbriae or *E. coli* LPS on dentine slices in 48-well culture plates for a further 7 days. After incubation, the cells were stripped by ultrasonication in 0.25 M ammonium hydroxide, and the dentine slices were immersed in hematoxylin to observe the resorption pits formed by osteoclasts under microscopy. Each dentine slice was measured using an Olympus image analysis system (Olympus Corporation, Tokyo, Japan). The results are expressed as the mean \pm standard deviation (SD) of triplicate cultures.

Cytokines: BALB/c mouse peritoneal macrophages were incubated in serum-free α -MEM in 24-well culture plates at 37°C for 1 hr in a humidified atmosphere of 5% CO₂ in air, and then, the culture plates were washed 3 times with α -MEM to remove non-adherent cells. The culture medium was then replaced with fresh α -MEM with or without various concentrations of *P. gulae* 41-kDa fimbriae and *E. coli* LPS, and then, culture was continued in triplicate for 6 hr. After incubation for 6 hr, the supernatants were collected and stored at -80°C until the assay for IL-1 β and TNF- α production. The levels of IL-1 β and TNF- α production in the samples were determined using an enzyme-linked immunosorbent assay (ELISA) kit system from Genzyme-Techne (Minneapolis, MN, U.S.A.). The results were determined using a standard curve prepared for each assay.

Animal experimental design: Twelve specific-pathogen-free (SPF) 3-week-old male Sprague-Dawley (SD) rats, each weighing 50 g, were obtained from a commercial farm (Japan SLC, Hamamatsu, Japan). Rats were divided randomly into 2 groups of 6. Group A served as the sham-infected control, and group B was the *P. gulae*-infected group. Each group was kept in a cage throughout the experiment. Rats

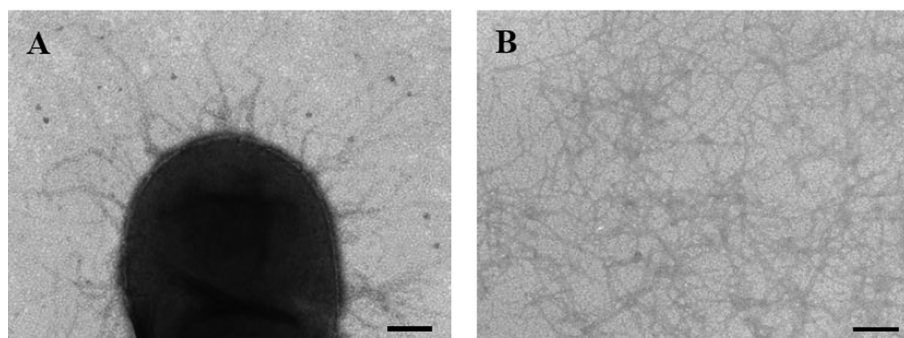


Fig. 1. Electron microscopy. Transmission electron micrographs of *P. gulae* ATCC 51700 (A) and purified 41-kDa fimbriae (B). Bars, 0.2 μ m.

were fed a standardized diet of hard briquettes and water and were maintained under a 12-hr light-dark cycle (lights on at 8:00 am) at a temperature of 22°C and a relative humidity of 50%. Rats were given sulfamethoxazole (1 mg/ml) and trimethoprim (200 μ g/ml) in drinking water for 7 days *ad libitum* to reduce the original oral flora, followed by a 3-day antibiotic-free period before *P. gulae* infection. They were orally infected with *P. gulae* suspended in 5% carboxymethylcellulose; each rat received 0.5 ml of the 5% carboxymethylcellulose (1.5×10^9 cells/ml) by oral gavage (5 times) at 48-hr intervals. Group B was orally infected with *P. gulae*. The experimental procedures of this study were reviewed and approved by the Committee on Ethics of Animal Experiments at Kanagawa Dental University and were performed under the guidelines for animal experimentation at Kanagawa Dental University.

Measurement of alveolar bone loss: Measurement of alveolar bone loss was assayed with the method of Hamada *et al.* [7] with some modifications. The left sides of the upper jaws were used as dry specimens to measure horizontal alveolar bone loss. Upper jaws were defleshed after 10 min in an autoclave at 15 pounds/inch² and then immersed in 3% hydrogen peroxide, rinsed and air-dried. Horizontal alveolar bone resorption around the maxillary molars was evaluated morphometrically. The distance between the cement-enamel junction (CEJ) and the alveolar bone crest (ABC) was measured at seven buccal sites per rat using a dissecting microscope ($\times 40$) fitted with a digital high-definition system (Digital HD microscope VH-7000; Keyence, Osaka, Japan) and standardized to provide measurements in millimeters. Results were expressed as the mean \pm standard error of 6 animals.

Recovery of *P. gulae*: To confirm *P. gulae* colonization, plaque samples were collected with a sterile cotton swab from the oral cavity on day 49 after *P. gulae* infection. Plaque samples in 100 μ l distilled water were boiled for 10 min, cooled on ice and examined by polymerase chain reaction (PCR). For the identification of *P. gulae*, primers were used to amplify a 451-base pair fragment of the 16S rRNA gene: 5'-TGCTTGGTTGCATGATCGG-3' and 5'-CAACGGCAGCTGAACG-3'. In brief, 5 μ l of the sample was added to 45 μ l of reaction mixture containing

5 μ l 10x PCR buffer, DNA polymerase and 0.2 mM of each deoxyribonucleotide. PCR amplification was performed in a thermal cycler, including an initial denaturation step at 95°C for 2 min followed by 36 cycles of denaturation at 95°C for 30 sec, primer annealing at 60°C for 1 min and extension at 72°C for 1 min, and then, a final elongation step was performed at 72°C for 2 min. Amplicons were detected by electrophoresis of 10 μ l PCR product on a 1.5% agarose gel. Electrophoresis was conducted at 10 V/cm in Tris-boric acid-ethylenediaminetetraacetic acid buffer. The gel was stained with 0.5 μ g/ml ethidium bromide and photographed under 302-nm ultraviolet light. Band sizes were confirmed with reference to molecular size markers.

Statistical analysis: Data comparisons between the 2 groups were analyzed by Student's *t*-test. Statistical differences between groups were tested by one-way ANOVA followed by Tukey's post hoc tests using StatView. A value of $P < 0.05$ was considered significant. Computations were performed using a statistical software programme (STATVIEW version 5.0; Abacus Concepts, Inc., Berkeley, CA, U.S.A.).

RESULTS

Isolation and purification of the 41-kDa fimbriae from *P. gulae*: Fimbrial structures were observed on the cell surface of *P. gulae* by transmission electron microscopy using the negative staining technique (Fig. 1A). Fimbrial protein was purified using DEAE-Sepharose CL-6B column chromatography and was detected as a major component of the main peak eluted at 0.15 M NaCl. The purified fimbrial preparation showed a single band of 41-kDa in SDS-PAGE under reducing conditions when stained with Coomassie Brilliant Blue R-250 (Fig. 2). Negative staining revealed that the purified protein was a dense network of fimbrial structures (Fig. 1B).

Osteoclast differentiation: To examine the level of osteoclast differentiation, a mouse co-culture system in the presence of various concentrations of purified 41-kDa fimbrial protein was used. Osteoclast differentiation was significantly enhanced with 41-kDa fimbrial protein treatment. The numbers of TRAP-positive multinucleated cells after treatment with 10.0 μ g/ml, 1.0 μ g/ml and 0.1 μ g/ml

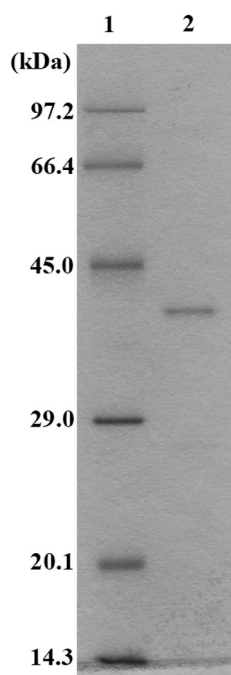


Fig. 2. SDS-PAGE analysis. SDS-PAGE of the purified 41-kDa fimbrial protein from *P. gulae* ATCC 51700. Lanes: 1, standard proteins; 2, purified 41-kDa fimbrial protein.

were 129.67 ± 8.18 , 144.67 ± 5.44 and 42.00 ± 4.32 , respectively (Fig. 3a). Bacterial cell wall components, such as LPS, may work as activators to induce osteoclast maturation. The 41-kDa fimbrial protein at concentrations of $10.0 \mu\text{g/ml}$ and $1.0 \mu\text{g/ml}$ induced the same level of osteoclast formation as *E. coli* LPS. The maximal osteoclast formation, induced by $1.0 \mu\text{g/ml}$ of 41-kDa fimbrial protein, was 4-fold higher than that of the non-stimulated control ($P < 0.01$) (Fig. 3b).

Pit formation: We examined the ability of 41-kDa fimbrial protein to activate bone resorption. Formation of pits was observed on all dentin surfaces. The 41-kDa fimbrial protein stimulated pit formation in a concentration-dependent manner (Fig. 4a). The total areas stimulated by the 41-kDa fimbrial protein at $10.0 \mu\text{g/ml}$ and $1.0 \mu\text{g/ml}$ were $5.79 \pm 0.77 \text{ mm}^2$ and $2.74 \pm 0.74 \text{ mm}^2$, respectively, which were significantly greater than that in the non-stimulated control ($P < 0.01$) (Fig. 4b).

Cytokines: The purified 41-kDa fimbrial protein induced IL-1 β and TNF- α production in BALB/c mouse peritoneal macrophages after 6 hr of incubation in a dose-dependent manner. Significant levels of IL-1 β production were observed, $177.42 \pm 5.74 \text{ pg/ml}$ and $81.37 \pm 7.65 \text{ pg/ml}$ following stimulation with the 41-kDa fimbrial protein at $10.0 \mu\text{g/ml}$ and $1.0 \mu\text{g/ml}$, respectively ($P < 0.01$). The level of TNF- α production induced by the 41-kDa fimbrial protein at $10.0 \mu\text{g/ml}$ was approximately the same as that induced by *E. coli* LPS. The level of TNF- α induced by 41-kDa fimbrial protein at $1.0 \mu\text{g/ml}$ was slightly lower than that induced by LPS, but the protein showed 25-fold higher production of

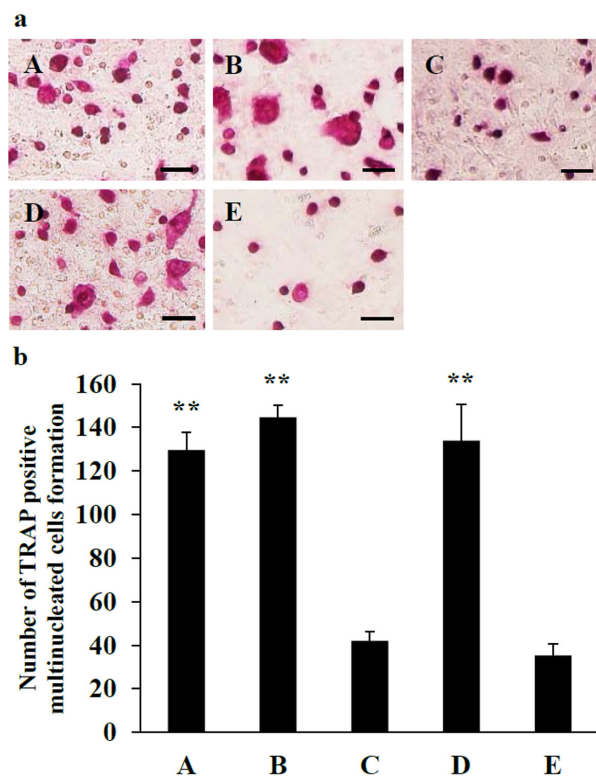


Fig. 3. Osteoclast differentiation. Different concentrations of the 41-kDa fimbrial protein were added to examine the differences in osteoclast differentiation (a). Bars, $50 \mu\text{m}$. TRAP-positive multinucleated cells (more than 3 nuclei/cell) were counted as osteoclasts. Number of TRAP-positive cells (b). ** $P < 0.01$. Ten (A), 1.0 (B) and $0.1 \mu\text{g/ml}$ (C) of 41-kDa fimbrial protein, $1.0 \mu\text{g/ml}$ of *E. coli* LPS (D) and negative control (E).

TNF- α as compared with that of the non-stimulated control ($P < 0.01$) (Table 1).

Alveolar bone loss: Apparent horizontal bone loss was observed in rats infected with *P. gulae*. The bone levels of the sham-infected group and *P. gulae*-infected group were $0.33 \pm 0.22 \text{ mm}$ and $0.42 \pm 0.27 \text{ mm}$, respectively (Fig. 5). At the termination of the experiments, *P. gulae* was detected by PCR from rats in the *P. gulae*-infected group. However, *P. gulae* could not be detected in noninfected rats (date not shown).

DISCUSSION

Periodontal disease is a significant oral problem, characterized by halitosis, gingival inflammation, increased periodontal pocket depth and alveolar bone loss, resulting in loosening and eventual loss of teeth [26]. Periodontitis in companion animals is an almost identical disease to that in humans in terms of disease course and clinical presentations. Black-pigmenting anaerobic bacteria have been isolated from the periodontal pockets of several wild animals [1, 4, 12]. The most frequently isolated black-pigmented an-

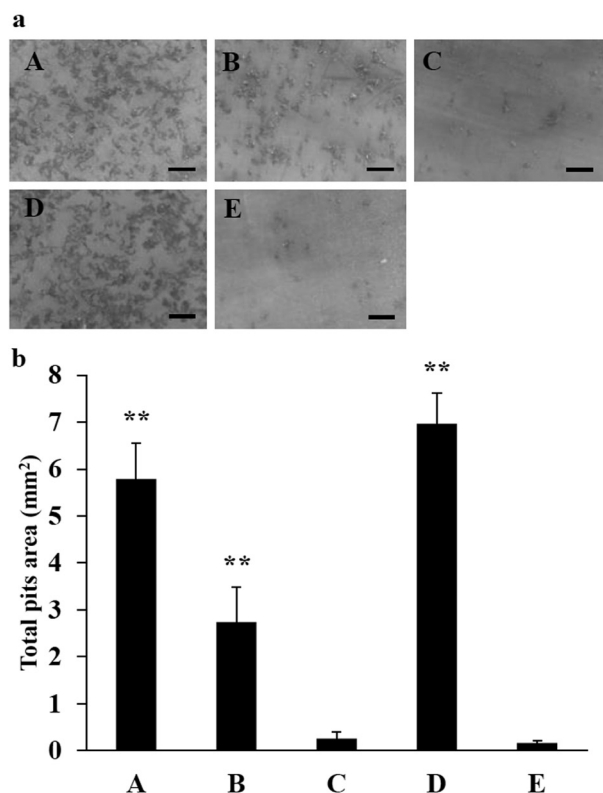


Fig. 4. Pit formation. Photographs of pit formation on the dentine slices stimulated by different concentrations of 41-kDa fimbrial protein (a). Bars, 50 μ m. The 41-kDa fimbrial protein apparently developed pit formation absorbed by osteoclast precursors on the dentine slices (b). ** $P < 0.01$. Ten (A), 1.0 (B) and 0.1 μ g/ml (C), 1.0 μ g/ml of *E. coli* LPS (D) and negative control (E).

Table 1. Induction of cytokine production in macrophages

Stimulant	Dose (μ g/ml)	Cytokine level (pg/ml)	
		IL-1 β	TNF- α
<i>P. gulae</i> 41-kDa fimbriae	10.0	177.42 \pm 5.74 ^a	2,095.23 \pm 5.53 ^a
	1.0	81.37 \pm 7.65 ^a	1,816.26 \pm 12.91 ^a
	0.1	15.09 \pm 13.39	631.29 \pm 33.18 ^a
<i>E. coli</i> LPS F583	1.0	281.59 \pm 3.83 ^a	2,087.41 \pm 23.97 ^a
None		15.09 \pm 9.57	72.04 \pm 5.53

The results are expressed as the mean \pm SD of triplicate cultures. a) Significantly different from values of the negative control ($P < 0.01$).

aerobic bacteria in canine periodontal pockets are *P. gulae*, *P. salivosa* and *P. denticanis* [1, 17]. Periodontal pathogens induce inflammatory reactions in the surrounding tissues [19].

The *fimA* gene encoding the 41-kDa fimbrial subunit protein (FimA) of *P. gulae* ATCC 51700 has been cloned by Hamada *et al.* [8]. Recently, Nomura *et al.* [25] reported that the *fimA* genes of 17 *P. gulae* strains had been sequenced and classified into 2 major types. In the present study, the 41-kDa fimbriae were isolated from *P. gulae* ATCC 51700

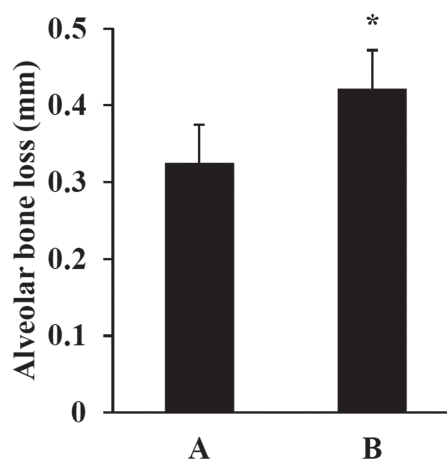


Fig. 5. Alveolar bone loss in rats by *P. gulae* infection. The distance from the CEJ to the ABC was measured at seven buccal sites per rat. Alveolar bone loss of the *P. gulae*-infected group was significantly greater than that of the sham-infected group. * $P < 0.05$. Sham (A) and *P. gulae*-infected (B).

and subjected to further purification on a DEAE-Sepharose CL-6B column. The fractions collected were observed as a single band on SDS-PAGE (Fig. 2). We examined the effects of the 41-kDa fimbrial protein from *P. gulae* on the induction of osteoclast differentiation and on inflammatory cytokine production in murine peritoneal macrophages. Osteoclasts, which are differentiated into multinuclear cells derived from hemopoietic precursor cells, are responsible for bone resorption [30]. LPS is a major component of the cell wall of Gram-negative bacteria. It has been reported to strongly induce inflammation, which is considered to cause bone destruction [15]. Our results suggest that the 41-kDa fimbrial protein has similar characteristics to LPS in osteoclast formation (Fig. 3). Therefore, 41-kDa fimbrial protein may have the ability to activate osteoclast formation in periodontal diseases.

The stimulation of the osteoclast precursor by the 41-kDa fimbrial protein on dentin slices resulted in a large absorption area in comparison with that in the non-stimulated control (Fig. 4). The 41-kDa fimbrial protein is capable of inducing bone resorption. Pit formation on dentin or bone slices is the most reliable evidence of osteoclast resorption activity [16]. The 41-kDa fimbrial protein also induced IL-1 β and TNF- α production from BALB/c mouse peritoneal macrophages (Table 1).

These inflammatory cytokines have been reported to stimulate osteoclast differentiation and alveolar bone loss through the induction of RANKL expression in osteoblasts [31]. Immune cells, mainly macrophages, infiltrate into the periodontal tissue and produce inflammatory cytokines [21, 31], such as prostaglandin E₂, IL-1, IL-6 and TNF- α . Roodman *et al.* [28] reported that both IL-1 and TNF- α were potent stimulators of bone resorption *in vitro* and *in vivo*. In humans, IL-1 β expression was elevated in gingival crevicular fluid at sites of recent bone and attachment loss in

patients with periodontal disease [23].

Rats orally infected with *P. gulae* exhibited significant alveolar bone loss as compared with that of the sham-infected rats (Fig. 5). At the termination of the experiments, *P. gulae* DNA was detected by PCR from rats in the *P. gulae*-infected group. This finding indicates that *P. gulae* is capable of adhering to and colonizing the oral cavity. Umemoto *et al.* [33] demonstrated the importance of FimA of *P. gingivalis* in adherence of the protein to oral cells and in alveolar bone loss in rats. It has been shown that *P. gingivalis* wild-type was more adherent to human gingival epithelial cells than *P. gingivalis* fimA knockout mutant MPG1. In addition, the mean CEJ to ABC distance of rats infected with the MPG1 strain was significantly reduced as compared with that of rats infected with the wild-type strain. The contribution of *P. gingivalis* to alveolar bone loss seems to be supported by the stimulation of osteoclasts, which induces bone destruction and inhibits bone formation [14]. The 41-kDa fimbriae of *P. gingivalis* appear as long filamentous structures and have been reported to induce inflammatory cytokines in human gingival fibroblasts and murine peritoneal macrophages and to promote the adherence of the organism to host tissues [10, 11]. In the current study, 41-kDa fimbriae of *P. gulae* stimulated osteoclast formation and function. The 41-kDa fimbrial protein is one of the components responsible for cytokine induction, and it thereby may stimulate the inflammatory destruction and alteration of gingival connective tissues and the loss of alveolar bone. Indeed, the *P. gulae* ATCC 51700 showed greater periodontal tissue destruction than that which occurred in the sham-infected rats. Consequently, the *P. gulae* fimbriae may play an important role in the induction of periodontal diseases.

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REFERENCES

- Allaker, R. P., de Rosayro, R., Young, K. A. and Hardie, J. M. 1997. Prevalence of *Porphyromonas* and *Prevotella* species in the dental plaque of dogs. *Vet. Rec.* **140**: 147–148. [Medline] [CrossRef]
- Amano, A. 2010. Bacterial adhesins to host components in periodontitis. *Periodontol.* **2000** **52**: 12–37. [Medline] [CrossRef]
- Amano, A., Nakagawa, I., Okahashi, N. and Hamada, N. 2004. Variations of *Porphyromonas gingivalis* fimbriae in relation to microbial pathogenesis. *J. Periodontol. Res.* **39**: 136–142. [Medline] [CrossRef]
- Fournier, D., Mouton, C., Lapiere, P., Kato, T., Okuda, K. and Menard, C. 2001. *Porphyromonas gulae* sp. nov., an anaerobic, Gram-negative coccobacillus from the gingival sulcus of various animal hosts. *Int. J. Syst. Evol. Microbiol.* **51**: 1179–1189. [Medline] [CrossRef]
- Genco, C. A., Van, D. T. and Amar, S. 1998. Animal models for *Porphyromonas gingivalis*-mediated periodontal disease. *Trends Microbiol.* **6**: 444–449. [Medline] [CrossRef]
- Hajishengallis, G., Wang, M., Liang, S., Liang, S., Shakhathreh, M. A., James, D., Nishiyama, S., Yoshimura, F. and Demuth, D. R. 2008. Subversion of innate immunity by periodontopathic bacteria via exploitation of complement receptor-3. *Adv. Exp. Med. Biol.* **632**: 203–219. [Medline]
- Hamada, N., Watanabe, K., Tahara, T., Nakazawa, K., Ishida, I., Shibata, Y., Kobayashi, T., Yoshie, H., Abiko, Y. and Umemoto, T. 2007. The r40-kDa outer membrane protein human monoclonal antibody protects against *Porphyromonas gingivalis*-induced bone loss in rats. *J. Periodontol.* **78**: 933–939. [Medline] [CrossRef]
- Hamada, N., Takahashi, Y., Watanabe, K., Kumada, H., Oishi, Y. and Umemoto, T. 2008. Molecular and antigenic similarities of the fimbrial major components between *Porphyromonas gulae* and *P. gingivalis*. *Vet. Microbiol.* **128**: 108–117. [Medline] [CrossRef]
- Hamada, S., Amano, A., Kimura, S., Nakagawa, I., Kawabata, S. and Morisaki, I. 1998. The importance of fimbriae in the virulence and ecology of some oral bacteria. *Oral Microbiol. Immunol.* **13**: 129–138. [Medline] [CrossRef]
- Hanazawa, S., Hirose, K., Ohmori, Y., Amano, S. and Kitano, S. 1988. *Bacteroides gingivalis* fimbriae stimulate production of thymocyte-activating factor by human gingival fibroblasts. *Infect. Immun.* **56**: 272–274. [Medline]
- Hanazawa, S., Murakami, Y., Hirose, K., Amano, S., Ohmori, Y., Higuchi, H. and Kitano, S. 1991. *Bacteroides (Porphyromonas) gingivalis* fimbriae activate mouse peritoneal macrophages and induce geneexpression and production of interleukin-1. *Infect. Immun.* **59**: 1972–1977. [Medline]
- Hardham, J., Dreier, K., Wong, J., Sfintescu, C. and Evans, R. T. 2005. Pigmented-anaerobic bacteria associated with canine periodontitis. *Vet. Microbiol.* **106**: 119–128. [Medline] [CrossRef]
- Harvey, C. and Emily, P. P. 1993. Hyperplastic gingivitis. p. 104. *In: Small Animal Dentistry* (Harvey, C. E. and Emily, P. P. eds.), Mosby-Year Books, St. Louis.
- Holt, S. C. and Thomas, E. B. 1991. Factors in virulence expression and their role in periodontal disease pathogenesis. *Crit. Rev. Oral Biol. Med.* **2**: 177–281. [Medline]
- Hotokezaka, H., Sakai, E., Ohara, N., Hotokezaka, Y., Gonzales, C., Matsuo, K., Fujimura, Y., Yoshida, N. and Nakayama, K. 2007. Molecular analysis of RANKL-independent cell fusion of osteoclast-like cells induced by TNF- α , lipopolysaccharide, or peptidoglycan. *J. Cell. Biochem.* **101**: 122–134. [Medline] [CrossRef]
- Hughes, D. E., Wright, K. R., Uy, H. L., Sasaki, A., Yoneda, T., Roodman, G. D., Mundy, G. R. and Boyce, B. F. 1995. Bisphosphonates promote apoptosis in murine osteoclasts *in vitro* and *in vivo*. *J. Bone Miner. Res.* **10**: 1478–1487. [Medline] [CrossRef]
- Isogai, H., Kosako, Y., Benno, Y. and Isogai, E. 1999. Ecology of genus *Porphyromonas* in canine periodontal disease. *Zentralbl. Veterinarmed. B* **46**: 467–473. [Medline]
- Kawata, Y., Hanazawa, S., Amano, S., Murakami, Y., Matsumoto, T., Nishida, K. and Kitano, S. 1994. *Porphyromonas gingivalis* fimbriae stimulate bone resorption *in vitro*. *Infect. Immun.* **62**: 3012–3016. [Medline]
- Koide, M., Kinugawa, S., Takahashi, N. and Udagawa, N. 2010. Osteoclastic bone resorption induced by innate immune responses. *Periodontol.* **2000** **54**: 235–246. [Medline] [CrossRef]
- Lamont, R. J. and Jenkinson, H. F. 1998. Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis*. *Microbiol. Mol. Biol. Rev.* **62**: 1244–1263. [Medline]
- Lamont, R. J. and Jenkinson, H. F. 2000. Subgingival colonization by *Porphyromonas gingivalis*. *Oral Microbiol. Immunol.* **15**: 341–349. [Medline] [CrossRef]
- Landi, L., Amar, S., Polins, A. S. and Van, D. T. 1997. Host mechanisms in the pathogenesis of periodontal disease. *Curr.*

- Opin. Periodontol.* **4**: 3–10. [Medline]
23. Lee, H. J., Kang, I. K., Chung, C. P. and Choi, S. M. 1995. The subgingival microflora and gingival crevicular fluid cytokines in refractory periodontitis. *J. Clin. Periodontol.* **22**: 885–890. [Medline] [CrossRef]
 24. Nakagawa, I., Inaba, H., Yamamura, T., Kato, T., Kawai, S., Ooshima, T. and Amano, A. 2006. Invasion of epithelial cells and proteolysis of cellular focal adhesion components by distinct types of *Porphyromonas gingivalis* fimbriae. *Infect. Immun.* **74**: 3773–3782. [Medline] [CrossRef]
 25. Nomura, R., Shirai, M., Kato, Y., Murakami, M., Nakano, K., Hirai, N., Mizusawa, T., Naka, S., Yamasaki, Y., Matsumoto-Nakano, M., Ooshima, T. and Asai, F. 2012. Diversity of fimbriolin among *Porphyromonas gulae* clinical isolates from Japanese dogs. *J. Vet. Med. Sci.* **74**: 885–891. [Medline] [CrossRef]
 26. Ozaki, K. and Hanazawa, S. 2001. *Porphyromonas gingivalis* fimbriae inhibit caspase-3-mediated apoptosis of monocytic THP-1 cells under growth factor deprivation via extracellular signal-regulated kinase-dependent expression of p21 Cip/WAF1. *Infect. Immun.* **69**: 4944–4950. [Medline] [CrossRef]
 27. Penman, S. and Harvey, C. E. 1990. Periodontal disease. pp. 37–39. In: Manual of Small Animal Dentistry (Harvey, C. E. and Orr, H. S. eds.), B.S.A.V.A., Cheltenham.
 28. Roodman, G. D., Ibbotson, K. J., MacDonald, B. R., Kuehl, T. J. and Mundy, G. R. 1985. 1,25-Dihydroxyvitamin D₃ causes formation of multinucleated cells with several osteoclast characteristics in cultures of primate marrow. *Proc. Natl. Acad. Sci. U.S.A.* **82**: 8213–8217. [Medline] [CrossRef]
 29. Sarkiala, E. M., Asikainen, S. E. A., Kanervo, A., Junttila, J. and Jousimies-Somer, H. R. 1993. The efficacy of tinidazole in naturally occurring periodontitis in dogs: bacteriological and clinical results. *Vet. Microbiol.* **36**: 273–288. [Medline] [CrossRef]
 30. Tani-Ishii, N., Penninger, J. M., Matsumoto, G., Teranaka, T. and Umemoto, T. 2002. The role of LFA-1 in osteoclast development induced by co-cultures of mouse bone marrow cells and MC3T3-G2/PA6 cells. *J. Periodontal Res.* **37**: 184–191. [Medline] [CrossRef]
 31. Taubman, M. A., Valverde, P., Han, X. and Kawai, T. 2005. Immune response: the key to bone resorption in periodontal disease. *J. Periodontol.* **76**: 2033–2041. [Medline] [CrossRef]
 32. Udagawa, N., Takahashi, N., Akatsu, T., Sasaki, T., Yamaguchi, A., Kodama, H., Martin, T. J. and Suda, T. 1989. The bone marrow-derived stromal cell lines MC3T3-G2/PA6 and ST2 support osteoclast-like cell differentiation in cocultures with mouse spleen cells. *Endocrinology* **125**: 1805–1813. [Medline] [CrossRef]
 33. Umemoto, T. and Hamada, N. 2003. Characterization of biologically active cell surface components of a periodontal pathogen. The roles of major and minor fimbriae of *Porphyromonas gingivalis*. *J. Periodontol.* **74**: 119–122. [Medline] [CrossRef]
 34. Weinberg, M. A. and Bral, M. 1999. Laboratory animal models in periodontology. *J. Clin. Periodontol.* **26**: 335–340. [Medline] [CrossRef]
 35. Yilmaz, O., Watanabe, K. and Lamont, R. J. 2002. Involvement of integrins in fimbriae-mediated binding and invasion by *Porphyromonas gingivalis*. *Cell. Microbiol.* **4**: 305–314. [Medline] [CrossRef]
 36. Zambon, J. J. 1996. Periodontal diseases: microbial factors. *Ann. Periodontol.* **1**: 879–925. [Medline] [CrossRef]
 37. van Winkelhoff, A. J. and de Graaff, J. 1991. Microbiology in the management of destructive periodontal disease. *J. Clin. Periodontol.* **18**: 406–410. [Medline] [CrossRef]