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Porphyromonas salivosa ATCC 49407 fimbriae induced osteoclast differentiation and cytokine production

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Abstract

Porphyromonas salivosa* ATCC 49407 is a black-pigmented, anaerobic, Gram-negative, and rod-shaped organism. *P. salivosa* is isolated from the gingival sulcus of various animals including dogs and cats. Fimbriae are filamentous components on the cell surface and are thought to play an important role in the colonization of periodontal tissues. We examined the involvement of the fimbrial protein in osteoclast differentiation and cytokine production in murine macrophages. Furthermore, alveolar bone resorption induced by *P. salivosa* infection in rats was evaluated. Fimbrial protein was purified from *P. salivosa* by selective protein precipitation and chromatography on a DEAE CL-6B anion exchange column. Western blotting analysis was performed with PAbs against fimbrial protein from *P. salivosa*. Expression of fimbriae on the surface of *P. salivosa* was investigated using transmission electron microscopy. To estimate osteoclast differentiation, bone marrow cells and MC3T3-G2/PA6 cells were cultured with or without the purified fimbrial protein. BALB/c mouse peritoneal macrophages were stimulated with the purified fimbrial protein, and cytokine production was determined by ELISA. Sprague-Dawley rats were infected with *P. salivosa*. Forty-five days after the last infection, the periodontal bone levels were determined by a morphometric measurement. We determined *P. salivosa* had fimbrial structure on the cell surface, and purified 60-kDa fimbrial protein. Osteoclast differentiation was significantly enhanced with the treatment of the 60-kDa fimbrial protein. The purified fimblial protein induced IL-1β and TNF-α production. Rats orally infected with *P. salivosa* exhibited significant bone loss compared with that of sham-infected rats. These results suggest that *P. salivosa* 60-kDa fimbriae may provoke an inflammatory response in host and be involved in periodontal tissue breakdown.

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Introduction

Porphyromonas salivosa is a black-pigmented, asaccharolytic, anaerobic, non-motile, non-spore-forming, Gram-negative, and rod-shaped organism. It is often isolated from the gingival sulcus of a number of animals, including dogs and cats[1-3)].

Previous studies reported that the etiology and pathology of periodontal disease in companion animals were similar to those of human periodontal disease. The majority of dogs and cats have periodontitis, which becomes more severe with age[4-6)]. Black-pigmented anaerobic bacteria has been implicated as the primary periopathogen. *Porphyromonas salivosa*, *Porphyromonas denticanis* and *Porphyromonas gulae*

were found to be the most frequently isolated black-pigmented anaerobic bacteria associated with canine periodontitis[7]. Norris *et al*.[8] reported that the strong positive correlation between *P. salivosa* and the grade of periodontal disease at canine sites strongly supported *P. salivosa* playing an important role in canine periodontal disease. Furthermore, *P. salivosa* is the same species as *P. macacae* isolated from monkeys[9, 10].

P. gingivalis is a pathogen that causes periodontal disease, a typical chronic inflammatory disease[11-14]. *P. gingivalis* fimbriae are important cell structures that contribute to adherence to and the invasion of host cells[15-18], and induce inflammatory processes in periodontal tissues through a number of mechanisms[19-21]. Furthermore, Ozaki *et al*.[22] reported that fimbriae function as virulence factors in inflammatory reactions because they stimulated the production of inflammatory cytokines by macrophages and fibroblasts.

Cytokines such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor-α (TNF-α) called bone resorptive cytokines because of their ability to promote bone resorption[23]. These cytokines may induce the expression and promote the solubilization of RANKL through osteoclast-supporting cells, osteoblasts, and stromal cells. Previous studies suggested that soluble RANKL affects not only osteoclast differentiation, but also cell migration and survival extension[24-26]. Moreover, Hamada *et al*.[27] reported that *P. gingivalis* 67-kDa fimbriae induced the expression of IL-1α, IL-1β, IL-6, and TNF-α in mouse peritoneal macrophages. These findings indicate that fimbriae play an important role in the induction of inflammatory responses in periodontal disease and alveolar bone loss.

Koyata *et al*.[28] showed that 60-kDa fimbriae were present on the surface of *P. salivosa* and also that the N-terminal amino acid sequence of the 60-kDa fimbrial protein of *P. salivosa* clearly differed from previously reported fimbrial proteins. However, the role of *P. salivosa* fimbriae in periodontal disease remains unclear. In the present study, we investigated the effects of the fimbrial protein from *P. salivosa* on the induction of osteoclast differentiation and cytokine production in murine macrophages.

Materials and Methods

1. Strains and cultivation conditions

P. salivosa ATCC 49407 was incubated anaerobically (15% CO_2, 15% H_2, and 70% N_2) (ANX-1; HIRASAWA, Tokyo, Japan) at 37°C in brain heart infusion (BHI) broth (Becton Dickinson Co., NJ, U.S.A.) supplemented with 0.5% yeast extract (Becton Dickinson Co.), 5 μg/ml hemin (Wako, Osaka, Japan), and 0.5 μg/ml vitamin K_1 (Wako).

2. Isolation and purification of 60-kDa fimbriae from *P. salivosa* ATCC 49407

The method of Yoshimura *et al*.[29] was employed to purify 60-kDa fimbriae. *P. salivosa* was incubated anaerobically for 18 hr in BHI broth. The bacterial cell pellet was harvested by centrifugation at 8,000 rpm at 4°C for 20 min and washed twice with 20 mM Tris-HCl buffer (pH 8.0) by repeated pipetting. The suspension was subjected to ultrasonication with a 3-mm microtip (Branson Ultrasonics Corporation, Danbury, CT, U.S.A.) and an output power of 25 W on the pulse setting with 3 cycles of 5 min in an icebox. The suspension of the sonic extract was centrifuged at 16,000 rpm at 4°C for 15 min and subjected to 40% ammonium sulfate saturation by the stepwise addition of ammonium sulfate. The precipitated protein was collected by centrifugation at 16,000 rpm at 4°C for 8 min, 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)-Sepharose CL-6B (GE Healthcare Bio-Sciences, Pittsburgh, PA, U.S.A.) column 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 to 0.3 M NaCl (Wako). The protein contents of the fractions were measured by ultraviolet light adsorption at 280 nm. Lipopolysaccharide (LPS) was not detected on silver-stained gels of the same preparation by a Silver Stain II kit (Wako). The endotoxicity of the fimbrial protein was not detected by a colorimetric Limulus amoebocyte lysate assay (GenScript, Tokyo, Japan).

3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein extracts were heated at 100°C for 10 min in loading buffer. Samples were applied to 12% polyacrylamide slab gels with a 4% stacking gel and electrophoresed at a constant current of 30 mA for 1 hr. Proteins were stained with Coomassie brilliant blue R-250 (Wako). A precision plus protein™ standards dual color (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.) was used for molecular weight calibrations.

4. Polyclonal antibodies

Polyclonal antibodies (PAbs) against the 60-kDa fimbrial protein were prepared using the purified protein described above as an immunogen. BALB/c mice (Nihon SLC, Inc., Shizuoka, Japan) were subcutaneously injected at multiple sites with 50 μg of the 60-kDa fimbrial protein emulsified with Freund's incomplete adjuvant (Becton Dickinson Co.). After 2 weeks, mice were injected weekly for 4 weeks with the immunogen. Each mouse was bled after the last booster injection, and antibodies were tested against the corresponding antigen by Western blotting. After an adequate antibody titer was obtained, mice were bled by cardiac puncture and sera were prepared and stored at −20°C. The experimental procedures of the present study were reviewed and approved by the Committee of Ethics on Animal Experiments of Kanagawa Dental University (Nos. 051, 17-026, and 17-051).

5. Western blotting

In the immunoblot analysis, proteins separated by 12% SDS-PAGE were transferred to a polyvinylidene difluoride membrane (Immun-Blot® PVDF membrane; Bio-Rad Laboratory) at 200 mA for 1 hr. Membranes were then treated with Tris-Buffer saline (TBS; 20 mM Tris-HCl pH 7.4, 0.5 M NaCl) containing 1% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, U.S.A.) to block unoccupied protein-binding sites. They were then incubated with PAbs specific for the 60-kDa fimbrial protein of *P. salivosa* ATCC 49407 at 37°C for 1 hr, washed in TBS-Tween (TBS with 0.5% Tween 20), incubated for 1 hr with goat anti-mouse immunoglobulin G conjugated with horseradish peroxidase (GE Healthcare), and then immersed in 4-chloro-1-naphthol (Sigma-Aldrich) solution to develop the color. The reaction was stopped by immersing the membranes in distilled water, and the membranes were then dried.

6. Electron microscopy

The fimbriae of *P. salivosa* cells were examined with a transmission electron microscope. Bacterial cells from an 18 hr anaerobic culture were collected by centrifugation (10,000 rpm for 1 min), washed, and resuspended (5×10^8 cells/ml) in phosphate-buffered saline (Nissui Pharmaceutical Co., Tokyo, Japan) (pH 7.4). Ten microliters of the cell suspension or purified fimbriae was applied to a copper grid coated with a thin Formvar film and air-dried. Samples were then negatively stained with 2% (wt./vol) uranyl acetate for 1 min, air-dried, examined, and photographed with a JEM-1220 electron microscope (JEOL Ltd., Tokyo, Japan) operating at 80 kV.

7. Detection of antibodies against 60-kDa fimbriae in cat serum

Twenty-five cats ranging in age 4 months to 16 years, both sexes (12 males, 13 females) and various breeds were included in the study. Cat serum was provided by Ogawa Animal Hospital in Yokosuka. Crude fimbrial proteins were separated by 12% SDS-PAGE and transferred to a PVDF membrane at 200 mA for 1 hr. The membranes were then treated with TBS containing 1% BSA (Sigma-Aldrich) to block unoccupied protein-binding sites.

Serum collected from cats acted on this membrane at 4°C overnight. After washing with TBS, membranes were incubated with goat anti-cat immunoglobulin G conjugated with horseradish peroxidase (Invitrogen, CA, U.S.A.) at 37°C for 1 hr, and then immersed in 4-chloro-1-naphthol (Sigma-Aldrich) to develop luminescence. The reaction was stopped by immersing the membranes in distilled water, and the membranes were then dried.

8. 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*.[30]. PA6 cells (1×10^6 cells/well), which function in a similar manner to stromal cells derived from bone marrow and bone marrow cells from BALB/c mouse (1×10^7 cells/well), were co-cultured in α-Minimum Essential Medium (α-MEM) containing 20% fetal bovine serum (FBS; Irvine Scientific, Santa Ana, CA, U.S.A.), 10^{-8} M dexamethasone (DEX; Sigma-Aldrich), RANKL (Sigma-Aldrich), and 10^{-8} M 1α,25 $(OH)_2D_3$ (calcitriol; Wako) with or without the *P. salivosa* 60-kDa fimbrial protein and *Escherichia coli* LPS F583 (Sigma-Aldrich). These cells were incubated in 48-well plates (Sumitomo Bakelite, Tokyo, Japan) at 37°C in 5% CO_2 for 7 days. After 7 days, 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-Aldrich), sodium tartrate (Sigma-Aldrich), and Fast Red Violet LB Salt (Sigma-Aldrich). TRAP-positive multinucleated cells containing three or more nuclei were counted as osteoclasts[31]. Cells without the *P. salivosa* 60-kDa fimbrial protein and *E.*

coli LPS were used as a negative control and positive control, respectively.

9. Pit formation

Pit formation was assayed using the method of Kawata *et al*.[32] with some modifications. 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 dishes (Corning Inc., Corning, NY, U.S.A.). Mouse osteoclast precursors were cultured with RANKL, DEX, calcitriol, and *P. salivosa* fimbriae or *E. coli* LPS in Osteo Assay Surface Multiple Well Plates (Corning Inc.) for a further 7 days. After the incubation, cells were stripped by ultrasonication in 10% bleach solution for 5 min and then washed twice with dH_2O. Each well was measured using an Olympus image analysis system (Olympus Corporation, Tokyo, Japan). The results obtained are expressed as the mean ± standard deviation (SD) of triplicate cultures.

10. 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% CO_2 in air, and culture plates were then 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. salivosa* 60-kDa fimbriae and *E. coli* LPS, and the culture was continued in triplicate for 6 hr. After being incubated for 6 hr, supernatants were collected and stored at −80°C until the assay for IL-1β and TNF-α production. The levels of IL-1β and TNF-α produced in the samples were measured using an enzyme-linked immunosorbent assay (ELISA) kit from Genzyme-Techne (Minneapolis, MN, U.S.A.). Results were obtained using a standard curve prepared for each assay.

11. 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 (Nihon SLC). Rats were divided randomly into 3 groups of 6. Group A served as the sham-infected control, group B was the *P. gingivalis*-infected group, and group C was the *P. salivosa*-infected group. Each group was kept in a cage throughout the experiment. Rats 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 relative humidity of 50%. Rats were given sulfamethoxazole (1 mg/ml) and trimethoprim (200 μg/ml) in drinking water for 7 days *ad libitum* to reduce the original oral flora, followed by a 3 days antibiotic-free period before being infected with *P. gingivalis* and *P. salivosa*. They were orally infected with *P. gingivalis* and *P. salivosa* suspended in 5% carboxymethylcellulose; each rat received 0.5 ml of 5% carboxymethylcellulose (1.5×10^9 cells/ml) by oral gavage (5 times) at 48 hr intervals. Group B was orally infected with *P. gingivalis* and Group C was orally infected with *P. salivosa*. The experimental procedures of the present 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.

12. Measurement of alveolar bone loss

Alveolar bone loss was measured using the method of Hamada *et al*.[33] 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/inch^2 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 alveolar bone crest (ABC) was measured at seven buccal sites per rat using a dissecting microscope (×40) fit 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 ± standard error of 6 animals.

13. Statistical analysis

Data comparisons between groups were performed by Tukey's tests. A value of $P<0.05$ was considered to be significant. Computations were performed using a statistical software program (STATVIEW version 5.0; Abacus Concepts, Inc., Berkeley, CA, U.S.A.).

Results

1. Isolation and purification of 60-kDa fimbriae from *P. salivosa*

Fimbrial structures were observed on the cell surfaces of *P. salivosa* by transmission electron microscopy

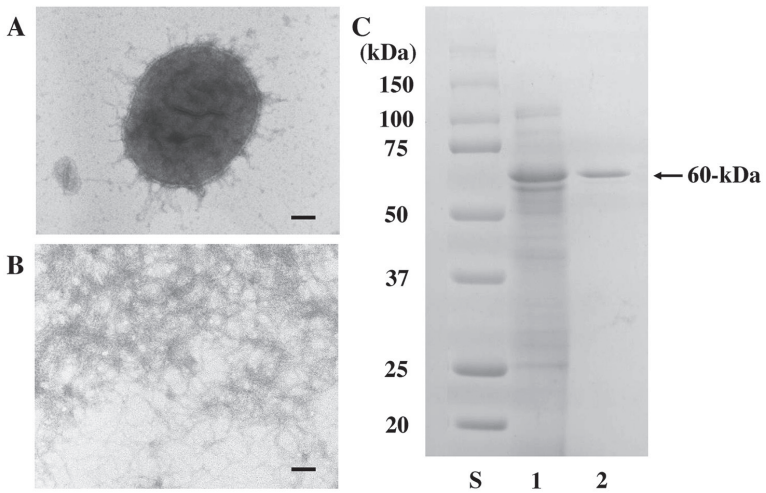

Figure 1. Purification of the fimbrial protein from *P. salivosa* ATCC 49407. *P. salivosa* ATCC 49407 had fimbriae on the cell surface (A). Electron micrograph of the purified 60-kDa fimbrial protein (B). SDS-PAGE of the purified 60-kDa fimbriae (C). Lanes: S, Standard proteins; 1, crude extract 2, purified protein. Bars, 0.2 μm.

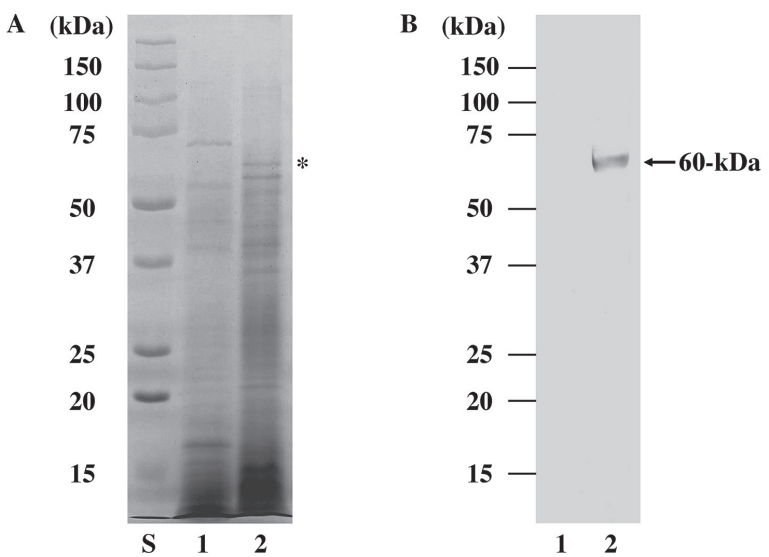

Figure 2. Immunoblot analysis of *P. salivosa* fimbriae. SDS-PAGE analysis of *Porphyromonas* spp. (A). SDS-PAGE was electrophoretically transferred to a membrane and incubated with 60-kDa PAbs (B). PAbs specific for 60-kDa fimbriae reacted strongly with *P. salivosa*. Lanes: S, Standard proteins; 1, *P. gingivalis* ATCC 33277; 2, *P. salivosa* ATCC 49407.

using the negative staining technique (Fig. 1A). The fimbrial protein was purified using DEAE-Sepharose CL-6B column chromatography and detected as a major component of the main peak eluted at 0.15 M NaCl. Negative staining revealed that the purified protein was a dense network of fimbrial structures (Fig. 1B). The purified fimbrial preparation showed a single band at 60-kDa on SDS-PAGE under reducing conditions when stained with Coomassie Brilliant Blue R-250 (Fig. 1C).

2. Immunoblot analysis of *P. salivosa* fimbriae

To confirm the presence of 60-kDa fimbriae in *P. salivosa* ATCC 49407 and *P. gingivalis* ATCC 33277, PAbs specific for the 60-kDa fimbrial protein were used. The protein corresponding to 60-kDa by SDS-PAGE was not confirmed in *P. gingivalis* ATCC 33277 (Fig. 2A). Western blotting confirmed that PAbs specific for the 60-kDa fimbrial protein only reacted with *P. salivosa*-purified 60-kDa fimbriae (Fig. 2B). In addition, no band reacting with whole cells of *P. gingivalis* ATCC

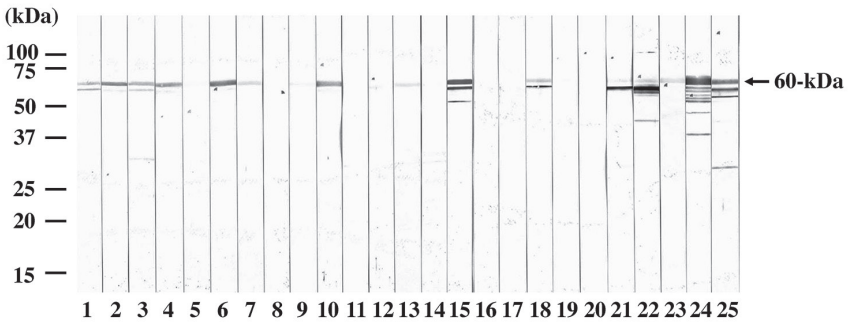

Figure 3. Western blot analysis of crude fimbriae using cat serum. Crude fimbrial proteins were electrophoresed on a 12% SDS polyacrylamide gel. The polyacrylamide gel was electrophoretically transferred to a PVDF membrane. Each strip was incubated with 25 domestic cat serum samples. The 60-kDa protein reacted with the majority of samples. However, sera of cats younger than one year old did not react with the 60-kDa fimbrial protein (8, 11, 17, 19, 20).

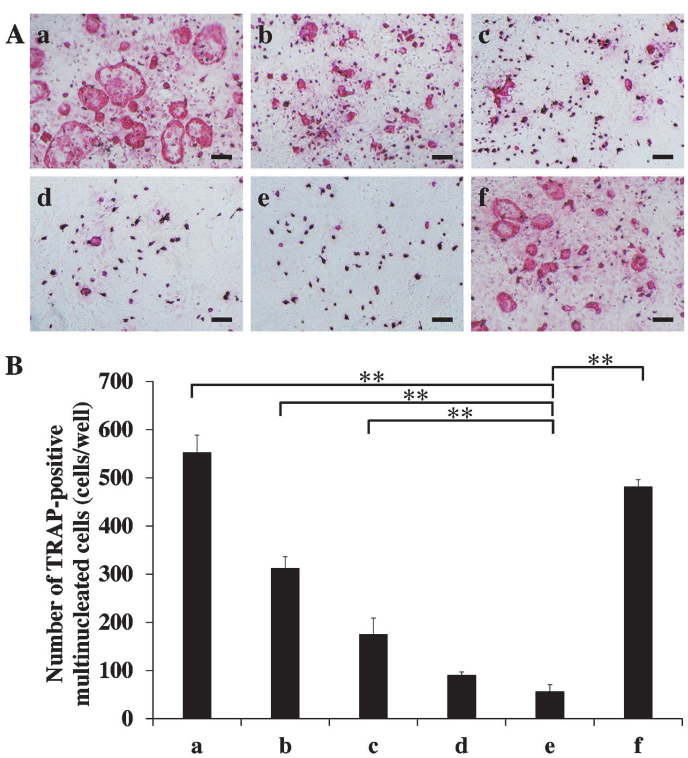

Figure 4. Osteoclast differentiation. Different concentrations of the *P. salivosa* 60-kDa fimbrial protein were added to examine the level of differentiation (A). *E. coli* LPS was used as a positive control. Osteoclast differentiation was significantly enhanced by the treatment with the 60-kDa fimbrial protein (B). 1.0 μg/ml (a), 0.1 μg/ml (b), 0.01 μg/ml (c), and 0.001 μg/ml (d) of the 60-kDa fimbrial protein, a negative control (e), and 1.0 μg/ml of *E. coli* LPS (f). Bars, 100 μm. $^{**}P<0.01$.

33277 was confirmed. Therefore, the antigenicity of the *P. salivosa* ATCC 49407 fimbrial protein differed from that of *P. gingivalis* ATCC 33277.

3. Detection of antibodies against 60-kDa fimbriae in cat serum

To confirm infection by *P. salivosa*, the presence or absence of antibodies was examined using 25 cat serum samples with the proteins from crude fimbrial fractions of *P. salivosa* ATCC 49407. The results obtained confirmed a reaction with a protein corresponding to 60-kDa in 17 out of 25 cats (Fig. 3). In cats aged 1 year or more, the positive rate was 85% (8 males and 9 females).

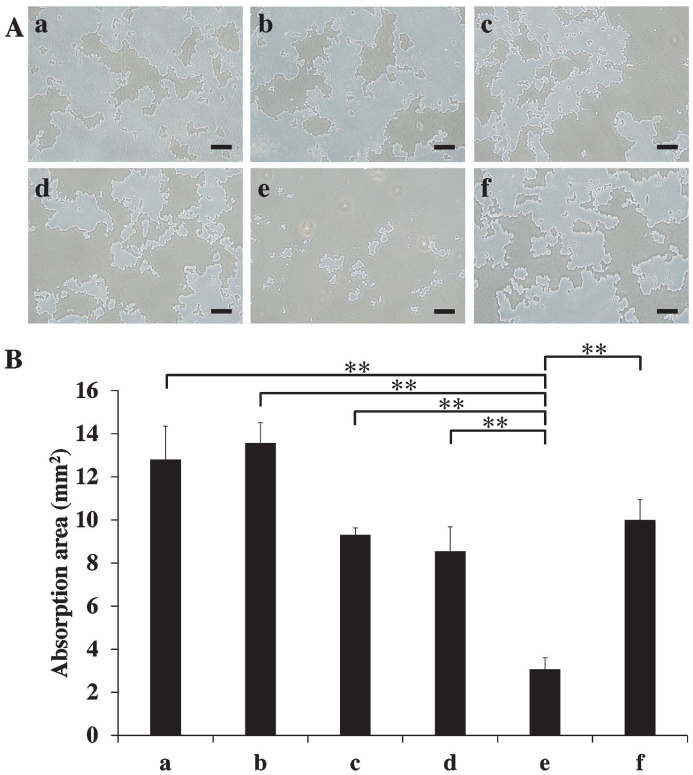

Figure 5. Pit formation. Absorption areas was significantly enhanced with treatment of the 60-kDa fimbrial protein. RANKL was used as a positive control (A). Formation of pits was observed on all osteo surfaces. The 60-kDa fimbrial protein stimulated pit formation in a concentration-dependent manner (B). 1.0 μg/ml (a), 0.1 μg/ml (b), 0.01 μg/ml (c), and 0.001 μg/ml (d) of the 60-kDa fimbrial protein, a negative control (e), and 30 ng/ml of RANKL (f). Bars, 200 μm. ***P*<0.01

4. Osteoclast differentiation

To examine the level of osteoclast differentiation, a mouse co-culture system in the presence of various concentrations of the purified 60-kDa fimbrial protein was used. Osteoclast differentiation was significantly enhanced by the 60-kDa fimbrial protein treatment. The numbers of TRAP-positive multinucleated cells after treatments with 1.0 μg/ml, 0.1 μg/ml, 0.01 μg/ml and 0.001 μg/ml were 551.67 ± 36.81, 312.00 ± 24.18, 174.67 ± 33.69, and 90.33 ± 7.36, respectively (Fig. 4A). Bacterial cell wall components, such as LPS, may function as activators to induce osteoclast maturation. The 60-kDa fimbrial protein at a concentration of 1.0 μg/ml induced the same level of osteoclast formation as *E. coli* LPS. Maximal osteoclast formation induced by 1.0 μg/ml of the 60-kDa fimbrial protein was 10-fold higher than that by the non-stimulated control (P<0.01) (Fig. 4B).

5. Pit formation

We examined the ability of the 60-kDa fimbrial protein to activate bone resorption. The formation of pits was observed on all dentin surfaces. The 60-kDa fimbrial protein stimulated pit formation in a concentration-dependent manner (Fig. 5A). The total areas stimulated by the 60-kDa fimbrial protein at 1.0 μg/ml, 0.1 μg/ml, 0.01 μg/ml and 0.001 μg/ml were 12.78 ± 1.57 mm^2, 13.55 ± 0.96 mm^2, 9.30 ± 0.33 mm^2 and 8.53 ± 1.15 mm^2, respectively, which were significantly larger than that by the non-stimulated control (P<0.01) (Fig. 5B).

6. Cytokines

The purified 60-kDa fimbrial protein induced IL-1β and TNF-α production in BALB/c mouse peritoneal macrophages after a 6 hr incubation in bone marrow cells and MC3T3-G2/PA6 cells. Significant production levels of IL-1β of 64.99 ± 5.64 pg/ml were observed following the stimulation with the 60-kDa fimbrial protein at 1.0 μg/ml (P<0.01). The production level of TNF-α induced by the 60-kDa fimbrial protein at 1.0 μg/ml was 2.5-fold higher than that induced by *E. coli* LPS. Significant production levels of TNF-α were observed; 4270.30 ± 45.45 pg/ml and 1076.91 ±

Table 1 Induction of cytokine production by macrophages

Stimulant	Dose (μg/ml)	Cytokine level (pg/ml)	
		IL-1β	TNF-α
P. salivosa 60-kDa fimbriae	1.0	64.99 ± 5.64	4270.30 ± 45.45
	0.1	11.21 ± 1.17	1076.91 ± 66.54
	0.01	0.00 ± 0.3	11.00 ± 1.41
E. coli LPS F583	1.0	161.55 ± 10.83	1683.73 ± 170.77
None		0.00 ± 0.61	0.00 ± 0.56

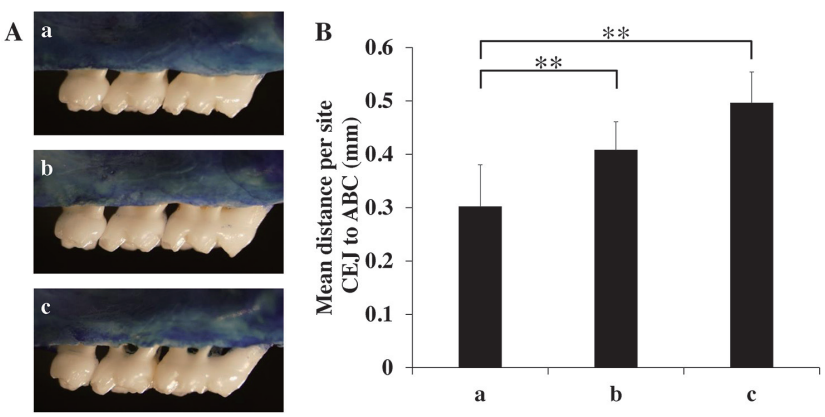

Figure 6. Alveolar bone loss in rats infected with *P. gingivalis* and *P. salivosa*. Horizontal bone loss around the maxillary molar (A). Changes in alveolar bone resorption caused by oral infection with *Porphyromonas* spp. (B). Values indicate mean bone loss levels with the standard error of the mean (n=6/group). Control (a), *P. gingivalis*-infected (b), and *P. salivosa*-infected (c). $^{**}P<0.01$.

66.54 pg/ml following the stimulation with the 60-kDa fimbrial protein at 1.0 μg/ml and 0.1 μg/ml, respectively ($P<0.01$) (Table 1).

7. Alveolar bone loss

Apparent horizontal bone loss was observed in rats infected with *P. gingivalis* and *P. salivosa* (Fig. 6A). The bone levels of the sham-infected group, *P. gingivalis*-infected group, and *P. salivosa*-infected group were 0.30 ± 0.08 mm, 0.41 ± 0.05 mm and 0.50 ± 0.06 mm, respectively (Fig. 6B). At the termination of experiments, *P. gingivalis* was detected by PCR in rats in the *P. gingivalis*-infected group. Similarly, *P. salivosa* was detected in the *P. salivosa*-infection group. However, *P. gingivalis* and *P. salivosa* were not detected in non-infected rats.

Discussion

The etiology and pathology of periodontal disease are characterized by inflammation of the gingiva, increases in periodontal pockets, attachment loss, and alveolar bone loss, resulting in the loosening and eventual loss of teeth. Periodontal disease in companion animals is similar to that in humans. Therefore, periodontal disease is considered to be a serious disease not only in humans, but also in companion animals. In recent years, some *Porphyromonas* spp., which are black-pigmented, anaerobic, non-motile, non-spore-forming, Gram-negative, and rod-shaped organisms, have been isolated from the periodontal pockets of several animals[1, 2, 7]. *P. salivosa* and *P. gulae* are often isolated from dogs and cats and have been implicated in periodontal disease in these animals. Previous studies reported that *P. gulae* isolated from dogs and their owners[34, 35]. These results suggest that several periodontopathic species could be transmitted between humans and their companion dogs. Therefore, it is important to investigate the pathogenic properties of *P. salivosa*. A previous study reported that the strong positive correlation between *P. salivosa* and the grade of periodontal disease at canine sites strongly supports *P. salivosa* playing an important role in canine periodontal disease[7]. Sasaki *et al*.[36] found that the 41-kDa fimbriae of *P. gulae*, a pathogenic bacterium of canine periodontal disease, induced osteoclast differentiation and cytokine production. However, the

pathogenicity of *P. salivosa* fimbriae remains unclear.

In the present study, we purified the *P. salivosa* fimbrial protein, and investigated the effects of the fimbrial protein from *P. salivosa* on the induction of osteoclast differentiation and cytokine production. Fimbrial structures were observed on the cell surface of *P. salivosa* ATCC 49407 by transmission electron microscopy (Fig. 1A), and the fimbrial protein was purified by selective protein precipitation and chromatography on a DEAE CL-6B anion exchange column (Fig. 1B). The purified fimbrial protein obtained on SDS-PAGE had a molecular weight of 60-kDa and was observed as a single band (Fig. 1C). SDS-PAGE and Western blotting using PAbs specific for the 60-kDa fimbrial protein of *P. salivosa* confirmed that this protein was not present in *P. gingivalis* (Fig. 2B). Therefore, the 60-kDa fimbrial protein clearly differed in molecular weight and antigenicity from the *P. gingivalis* fimbrial protein. Western blotting was used to confirm the reactivity of cat serum to the *P. salivosa* 60-kDa fimbrial protein. The results obtained confirmed that 17 out of 25 cats reacted with a protein corresponding to 60-kDa and the positive rate was 85% in cats aged 1 year or older (Fig. 3). Therefore, many cats were infected with *P. salivosa* with aging. *P. salivosa* fimbriae have been shown to induce inflammatory cytokine production because they stimulated the production of inflammatory cytokines by macrophages and fibroblasts[27, 37]. The 60-kDa fimbrial protein of *P. salivosa* induced osteoclast formation in a concentration-dependent manner ($P < 0.01$), and osteoclast differentiation ability was higher in 1.0 μg/ml of the 60-kDa fimbriae-stimulated group than in 1.0 μg/ml of the *E. coli* LPS 583-stimulated group (Fig. 4). Furthermore, pit formation was significantly enhanced by the addition of the 60-kDa fimbrial protein (Fig. 5) in a concentration-dependent manner ($P < 0.01$). These results suggest that *P. salivosa* 60-kDa fimbriae may cause alveolar bone loss in periodontal disease as well as *P. gingivalis* fimbriae and *P. gulae* fimbriae. Previous studies reported that inflammatory cytokines are involved in alveolar bone loss in periodontal disease[38, 39]. IL-1 and TNF-α are produced from monocytes and macrophages, and are known to induce osteoclast differentiation by enhancing osteoblast RANKL expression[40]. Furthermore, in the present study, the production of IL-1β and TNF-α by mouse macrophages was significantly induced by the addition of the 60-kDa fimbrial protein (Table 1). Alveolar bone loss was significantly greater in rats orally infected with *P. salivosa* than in sham-infected rats (Fig. 6). At the termination of experiments, *P. salivosa* DNA was detected by PCR from rats in the *P. salivosa*-infected group. This result indicates that *P. salivosa* adheres to and colonizes the oral cavity. Collectively, the present results suggest that *P. salivosa* 60-kDa fimbriae provoke inflammatory responses in hosts and are involved in the breakdown of periodontal tissue.

Conflict of interest statement

The authors declares that there is no conflict of interests regarding the publication of this paper.

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