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Heterogenic virulence and related factors among clinical isolates of *Porphyromonas gingivalis* with type II fimbriae

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Inaba H, Nakano K, Kato T, Nomura R, Kawai S, Kuboniwa M, Ishihara K, Ooshima T, Amano A. Heterogenic virulence and related factors among clinical isolates of Porphyromonas gingivalis *with type II fimbriae.*
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Background/aims: *Porphyromonas gingivalis* is a periodontal pathogen whose fimbriae are classified into six genotypes (types I–V and Ib) based on the diversity of the *fimA* genes encoding the fimbrial subunits. Accumulated evidence suggests that *P. gingivalis* strains with type II fimbriae are more virulent as compared to those with other types. However, it is unknown if strong virulence is uniformly conserved among clones with type II fimbriae. In the present study, we compared infectious inflammatory changes in clinical isolates of *P. gingivalis* with type II fimbriae using a mouse abscess model to examine their pathogenic heterogeneity and heterogeneity-related factors.
Methods: Suspensions of nine different clinical isolates with type II fimbriae were subcutaneously injected into female BALB/c mice and inflammatory parameters, such as serum sialic acid concentration, were compared.
Results: Many of the type II fimbrial isolates caused severe inflammation in the mice, though some were less causative, as was the control strain ATCC 33277 (type I fimbria strain). These results showed that pathogenic heterogeneity exists among *P. gingivalis* clones with type II fimbriae. Further, the heterogeneity-related factors of *P. gingivalis* strains were analyzed and the pathogenic potentials showed positive relationships to gingipain activities and invasive efficiency but not to hydrophobicity or autoaggregation. In addition, invasive efficiency was related to the activities of gingipains that were extracellularly secreted.
Conclusion: These results suggest that pathogenic heterogeneity has relationships with the invasive and proteolytic activities of *P. gingivalis* clones with type II fimbriae.

Key words: fimbriae; mouse model; *Porphyromonas gingivalis*; virulence

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Porphyromonas gingivalis, a Gram-negative black-pigmented anaerobe, is considered to be a bona fide pathogen that causes several forms of severe periodontal disease (52). This organism has also been reported to contribute to systemic conditions such as cardiovascular diseases (34) and preterm low birth weight (42). *P. gingivalis* expresses a number of potential virulence factors including fimbriae, as well as Arg-specific cysteine proteinases [gingipain A and B, (RgpA and RgpB, respectively)] and lysine-specific cysteine proteinase [Lys-gingipain (Kgp)], which contribute to the pathogenesis of periodontitis (22, 46). Various studies have demonstrated that fimbriae mediate the bacterial invasion of several human cell lines and assist *P. gingivalis* in its persistence in intracellular locations, which leads to further spreading into adjacent tissues (18, 24, 33, 54). Gingipains have also been reported to be major virulence factors that degrade various host proteins, leading to impaired cellular integrity and function (22, 46). These virulence potentials are suggested to be a pathogenetic paradigm of infection, in which *P. gingivalis* disrupts periodontal tissues.

The fact that *P. gingivalis* can be present in both periodontal pockets undergoing destruction and the healthy gingival margins has suggested its clonal hetero-

genicity, with subpopulations of high and low pathogenicities (9, 16, 17, 44). *P. gingivalis* fimbriae are classified into six genotypes (types I–V, and Ib) based on the different nucleotide sequences of the *fimA* genes encoding the fimbrial subunits (19, 37, 39). Previously, we developed a sensitive polymerase chain reaction (PCR) assay using *fimA* type-specific primer sets to differentiate fimbria genotypes among *P. gingivalis* clones harbored by periodontitis patients and periodontally healthy adults (3, 5), and a majority of the organisms isolated from the patients were found to carry type II fimbriae. Further, *P. gingivalis* with type II fimbriae was detected more frequently in the deeper periodontal pockets (6). A significant association of *P. gingivalis* clones carrying type II fimbriae with marginal periodontitis has also been reported by other studies with different cohorts conducted in Germany (10), Brazil (35), Switzerland (51), and China (53).

In another study, we compared the various virulence potentials of *P. gingivalis* strains with different fimbrial types using a mouse abscess model (41). Among the six types of fimbriae, type II strains caused the most significant induction of serum sialic acid (parameter of systemic inflammation), as well as other infectious symptoms, followed by types Ib, IV, and V. In contrast, types I and III caused only scant inflammatory changes. In addition, fimbria-deficient mutants of type II strains clearly lost their infectious abilities. Those findings suggested that type II fimbriae are a significant virulence factor of *P. gingivalis*. In addition, our other *in vitro* studies using cultured cells demonstrated that type II fimbriae are critical for the impairment of gingival epithelial cell function, as well as for the prevention of the homeostatic, regenerative, and healing properties of periodontal tissues (26, 40). Together, these findings strongly suggest that type II fimbriae are essential for *P. gingivalis* to initiate human marginal periodontitis, leading to tissue deterioration.

Although *P. gingivalis* clones with type II fimbriae have been clearly shown to be more virulent compared to other fimbria types, it remains to be elucidated if that strong pathogenicity is uniformly conserved as a typical characteristic among clones carrying type II fimbriae. In addition to fimbriae, *P. gingivalis* has a number of other potential virulence factors such as gingipains and capsules containing lipopolysaccharide, which are involved in the pathogenesis of periodontitis (22, 33, 46). Those other virulence factors may also contribute to the varying pathogenicity among type II fimbrial clones.

In the present study, we compared infectious inflammatory changes in a mouse abscess model to examine if pathogenic heterogeneity existed among different clinical isolates of *P. gingivalis* with type II fimbriae, and also investigated possible bacterial factors related to that heterogeneity.

Materials and methods

Bacterial culture

All procedures used in this study were approved by the Review Board of the Tokyo Dental College Ethical Committee. Written informed consent was obtained before sample collection of dental plaque. Eight clinical isolates of *P. gingivalis* (TDC strains) obtained from the periodontal pockets of patients were used in this study, while the clinical isolate *P. gingivalis* OMZ314 was a kind gift from Prof. van Winkelhoff (Academic Centre for Dentistry Amsterdam, the Netherlands). These nine isolates were confirmed to possess type II *fimA* genes and fimbriae, as described previously (5, 38). *P. gingivalis* ATCC33277 (representative strain of type I fimbriae) and the nine type II isolates were grown in trypticase soy broth (BBL, Sparks, MD) supplemented with yeast extract (1 mg/ml), menadione (1 μg/ml), and hemin (5 μg/ml) under an anaerobic atmosphere, as described previously (4). The expression level of the fimbrial subunit protein (fimbrillin) in each strain was examined by immunoblotting as described previously (41).

Cell culture

Immortalized human gingival epithelial cells (30) were kindly provided by Prof. Murakami (Osaka University, Japan). The cells were maintained in Humedia KB-2 (Kurabo, Osaka, Japan) at 37°C in 5% CO_2. Two hours before each infection assay, the culture medium for immortalized human gingival epithelial cells was changed to fresh Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St Louis, MO) with 10% fetal calf serum.

Mouse abscess model

All animal procedures and protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Osaka University Graduate School of Dentistry before the experiments. Female BALB/c mice (5 weeks old) were maintained in horizontal-flow cabinets, and provided with sterile food and water *ad libitum*. In all, 109 mice were randomly divided into 10 groups to examine inflammatory changes caused by *P. gingivalis* infection. At 40 days of age, a single site approximately 1 cm lateral from the midline on the dorsal surface was depilated, and 0.1 ml bacterial suspension (1×10^9 colony-forming units of a test strain) or phosphate-buffered saline (PBS) containing no bacterial cells was injected, as described previously (41). For quantitative evaluation of infectious inflammatory changes, serum sialic acid concentration, known as an appropriate parameter of systemic inflammation, was measured (47). Blood specimens (0.1 ml) were collected from an orbital vein on days 0, 1, 2, and 4 after bacterial infection, then centrifuged at 800 ***g*** for 10 min to separate the serum. Sialic acid concentrations in sera were quantified colorimetrically using a commercial kit (Kyokuto Sialic Acid Test Reagents; Kyokuto Pharmaceutical Industrial Co., Tokyo, Japan) according to the manufacturer's instructions. The mice were also monitored for signs and symptoms of infection, i.e. ruffled hair, abscess formation, and emergence of erosion, as described previously (41). The body weight was measured 2 days after the bacterial injection. All mice were sacrificed under ether anesthesia 14 days after infection, after which the spleens were extirpated and weighed. Spleen weights were standardized by body weight.

Preparation of membrane vesicles of *P. gingivalis*

P. gingivalis membrane vesicles were prepared according to the method of Hayashi et al. (20), with some modifications. Briefly, *P. gingivalis* cells from a 500-ml culture (3-day culture) were removed from the growth medium by two centrifugations at 8000 ***g*** for 30 min each at 4°C and filtration through a 500-ml filter system (pore size: 0.22 μm) (Corning Inc., Corning, NY). Membrane vesicles in the supernatant were collected by ultracentrifugation at 100,000 ***g*** for 1 h at 4°C, then suspended in 500 μl PBS and kept at −80°C until use. Protein concentrations were measured using a bicinchonimic acid (BCA) protein assay kit (Pierce, Rockford, IL) after sonication for 1 min.

Covalent coupling of membrane vesicles or BSA to fluorescent microsphere beads

Approximately 1.35×10^9 sulfate fluorescent microsphere beads (MSB; diameter

1.0 μm; Molecular Probes, Eugene, OR) were conjugated with membrane vesicles (100 μg) or bovine serum albumin (BSA, 100 μg; Sigma) in PBS at 4°C overnight, as described previously (24). To determine coupling efficiency, the amounts of bound protein were measured using a BCA protein assay kit (Pierce) before blocking with PBS containing BSA.

Fluorescence analysis of adhesion and invasion of MSB conjugated with membrane vesicles

Approximately 6×10^4 immortalized human gingival epithelial cells were seeded on coverslips in 24-well plates (Iwaki, Tokyo, Japan). The cells were then incubated with MSB conjugated with membrane vesicles (3×10^7 MSB per well) in DMEM containing 10% fetal calf serum for 6 h and then fixed with 4% paraformaldehyde in PBS, after which F-actin was stained with Oregon Green 488-conjugated phalloidin (Molecular Probes). To analyze and quantify the adhesion and invasion efficiencies of the MSB, a laser scanning confocal microscope (model LSM510; Carl Zeiss, Thornwood, NY) was used, as described previously (24). To analyze the distribution of the MSB that invaded cells, optical sections were obtained along the *z*-axis at 0.15-μm intervals (60 sections; thickness, 9 μm), and images of the *x–z* and *y–z* planes were reconstructed with the orthogonal section tool of the LSM510 software.

Assays for gingipains, hydrophobicity, and autoaggregation

The activities of *P. gingivalis* gingipains including Rgp and Kgp were determined using the synthetic substrates *t*-butyloxycarbonyl-L-leucylglycyl-L-arginine-4-metylcoumaryl-7-amide and *t*-butyloxycarbonyl-L-valyl-L-leucyl-L-lysine-4-metylcoumaryl-7-amide, respectively (Peptide Institute, Osaka, Japan), as described previously (25). Bacterial hydrophobicity was determined as described previously (45) and autoaggregation of *P. gingivalis* cells was turbidimetrically assayed, also as described previously (2). Briefly, bacterial suspensions were adjusted to an optical density at 550 nm (OD_{550}) of 0.4. After homogenizing the suspension by mild pipetting, the progress of autoaggregation was monitored in a cuvette equipped with a stirrer at 37°C at 550 nm. The OD_{550} value was reduced in association with autoaggregation of the bacterial cells, with the autoaggregation rate expressed as the reduced value of OD_{550} per minute ($-d_{abs}/d_t$).

Typing of *kgp* gene alleles of *P. gingivalis* isolates

A PCR typing assay was performed to classify the *kgp* gene alleles, as described previously (36). The primers used were as follows: HG66 F, 5′-TGCACGTTATGACGATTTCACAT-3′; HG66R, 5′-ACTTCCGAATGTATTGTGATCGG-3′; *kgp*F, 5′-ATGTATACTTTCCGTATGTCTGCTTCTTC-3′; W83R, 5′-AAGCGTCGTTACCCGTAGAAGA-3′; and 381R, 5′-ATCAGAAAGTTGTTCGGCGTG-3′.

Determination of capsular K-type of *P. gingivalis* isolates

Capsular K-typing of *P. gingivalis* was performed as described previously (1). Briefly, the 15.4-kb PG0106–PG0120 region, which is a capsular locus of *P. gingivalis* W83, was amplified using Reddy Load Extensor mix (buffer 2; ABgene, Epsom, UK) with the primers PG0106F3 (5′-ATTTCAGGGATGGGCAGAAG-3′) and PG0117R3 (5′-GCATCGAGTAAGAGCATCCG-3′). The amplified DNA was digested with *Bgl*II. In addition, the 5.6-kb PG0116–PG0120 region, which is also a capsular locus of *P. gingivalis* W83, was amplified with the primer pair PG0117F3 (5′-AGAAGGCTTCAAAGGTTGGG-3′) and PG0117R3 (noted above). In the PG0106–PG0120 region (15.4 kb), DNA of the K3-serotype was amplified to a 9-kb fragment. None of the K5-serotype strains were positive with this primer pair. Following restriction with *Bgl*II, the PCR product of the K1-type gave 10 bands of >0.3 kb, while that of the K2-type showed four bands of >0.3 kb. In the amplification of the PG0116–PG0120 region (5.6 kb), K^- gave a 4.5-kb fragment, whereas the products of the K1- and K4-types were 5.6 and 3 kb, respectively.

Statistical analyses

All data are presented as the mean ± SD, except for experiments with the mouse abscess model, in which they are presented as the mean ± SE. Statistical analyses were performed using an unpaired Student's *t*-test. Multiple comparisons were performed with Pearson's product-moment correlation coefficient using STAT VIEW software (SAS Institute, Cary, NC).

Results

Inflammatory changes caused by clinical isolates in a mouse abscess model

The inflammatory changes caused by the nine clinical isolates of *P. gingivalis* with type II fimbriae were compared using a mouse abscess model. Strain ATCC33277 (representative strain of type I fimbriae), which was previously shown to be significantly less virulent and cytopathic than strains with type II fimbriae (26, 40, 41), was also tested. Five of the isolates (TDC60, 117, 275, and 120, and OMZ314) caused serum sialic acid concentrations to increase to 180.5 to 198.42 mg/dl, which were significantly greater than the concentration following infection with ATCC33277 (103.9 mg/dl) (Table 1). Other inflammatory parameters (survival rate, outward symptoms, spleen swelling, and body weight loss) also indicated greater virulence of these isolates as compared to ATCC33277. Further, the inflammatory changes caused by three isolates (TDC59, 222, and 243) were more severe than that caused by ATCC33277. In contrast, TDC285 was found to be less virulent than ATCC33277. These results clearly suggest that pathogenic heterogeneity exists among *P. gingivalis* clones with type II fimbriae.

Heterogeneity-related factors of *P. gingivalis* clones with type II fimbriae

The amount of fimbriae on bacterial cells could be crucial for the expression of pathogenicity; however, immunoblotting revealed negligible differences in the expression level of fimbrillin protein among the tested strains (data not shown). Several other factors reportedly related to the virulence of *P. gingivalis* were evaluated to investigate heterogeneity-related factors. *P. gingivalis* organisms with type II fimbriae were previously shown to adhere to and invade gingival epithelial cells much more swiftly and efficiently than other types (26, 40). Thus, the adhesive abilities of the present isolates were assayed using MSB conjugated with membrane vesicles, and six isolates showed significantly greater adhesive and invasive abilities than ATCC33277, whereas three other isolates (TDC 222, 243, and 285) were not significantly different (Fig. 1).

The proteolytic activities of Rgp and Kgp were assayed using bacterial cell extracts and culture supernatant, respectively. These activities also varied considerably among the isolates, and the six that

Table 1. Infectious inflammation by *Porphyromonas gingivalis* isolates with type II fimbriae

Isolate	Survival rate	Maximum concentration of serum sialic acid (mg/dl)	Symptoms of surviving mice (no./total)			Spleen wt. (mg/g body wt.)	Body wt. (%)
			Ruffled hair	Abscess	Erosion		
TDC60	6/10	196.2 ± 11.2	6/6	0/6	6/6	15.17 ± 2.32	85.8 ± 1.5
TDC117	9/11	198.4 ± 9.4	9/9	3/9	4/9	11.67 ± 2.14	88.7 ± 1.5
TDC275	11/11	196.4 ± 6.7	8/11	7/11	0/11	6.57 ± 0.51	91.8 ± 1.6
OMZ314	10/10	183.0 ± 10.4	9/10	10/10	4/10	14.00 ± 0.93	96.2 ± 1.8
TDC120	10/10	180.50 ± 18.0	7/10	6/10	1/10	9.96 ± 1.41	95.9 ± 1.5
TDC59	10/10	148.6 ± 8.8	0/10	5/10	0/10	5.53 ± 3.12	98.5 ± 0.9
TDC222	7/7	130.1 ± 9.5	0/7	0/7	0/7	4.32 ± 0.35	100.4 ± 1.5
TDC243	10/10	117.2 ± 9.3	0/10	0/10	0/10	4.86 ± 0.41	97.7 ± 0.6
TDC285	10/10	92.8 ± 3.1	0/10	0/10	0/10	4.51 ± 0.14	100.5 ± 0.9
ATCC33277	10/10	103.9 ± 4.1	0/10	0/10	0/10	4.89 ± 0.21	102.2 ± 1.2
No infection	10/10	79.3 ± 2.8	0/10	0/10	0/10	4.10 ± 0.25	102.3 ± 0.8

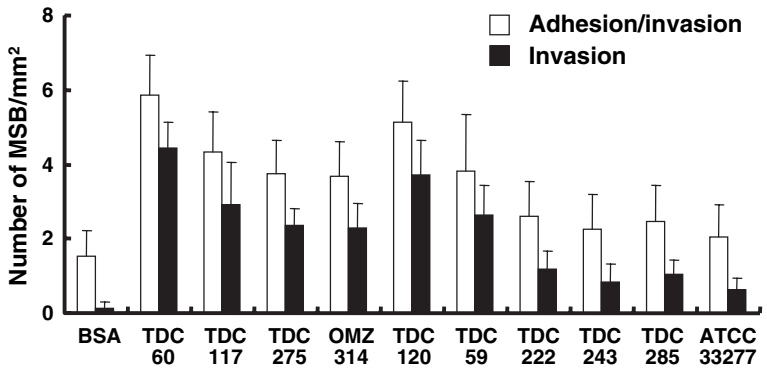

Fig. 1. Adhesion to and invasion of gingival epithelial cells by MSB conjugated with membrane vesicles of *Porphyromonas gingivalis* isolates with type II fimbriae. Approximately 6×10^4 gingival epithelial cells were incubated with MSB conjugated with membrane vesicles in DMEM containing 10% FCS for 6 h, after which cellular F-actin was stained with Oregon Green 488-conjugated phalloidin followed by fixation with 4% paraformaldehyde in PBS. To analyze and quantify the adhesion and invasion efficiencies of the MSB, a laser scanning confocal microscope was used. BSA was used as a control. ATCC33277 was included as a representative strain possessing type I fimbriae, with BSA as a negative control.

Table 2. Rgp and Kgp activities of *Porphyromonas gingivalis* isolates with type II fimbriae

Isolate	Cell extract		Culture supernatant	
	Rgp	Kgp (unit)	Rgp	Kgp (unit)
TDC60	15.74 ± 0.63	5.40 ± 0.16	22.48 ± 1.33	7.29 ± 0.25
TDC117	84.31 ± 0.86	18.31 ± 0.35	64.91 ± 7.03	8.91 ± 0.13
TDC275	30.97 ± 0.67	4.29 ± 0.11	23.46 ± 0.35	5.74 ± 0.12
OMZ314	22.52 ± 0.55	3.39 ± 0.05	19.70 ± 0.35	5.75 ± 0.15
TDC120	83.24 ± 0.64	17.36 ± 0.32	56.70 ± 2.52	8.15 ± 0.28
TDC59	27.42 ± 0.63	4.22 ± 0.15	45.94 ± 3.91	7.17 ± 0.28
TDC222	40.67 ± 3.74	6.39 ± 0.11	13.44 ± 2.02	4.95 ± 0.29
TDC243	8.63 ± 0.39	1.87 ± 0.01	13.62 ± 0.51	4.40 ± 0.06
TDC285	4.48 ± 0.11	1.02 ± 0.02	19.98 ± 1.69	5.15 ± 0.08
ATCC33277	21.31 ± 1.08	3.36 ± 0.36	18.76 ± 0.33	4.56 ± 0.12

caused increased serum sialic acid concentrations in the mouse model also showed greater activities of both cell-associated and secreted gingipains as compared to ATCC33277 (Table 2).

Autoaggregation of *P. gingivalis* mediates biofilm formation, while cell surface hydrophobicity is related to bacterial interaction with host cells (27). Kgp of *P. gingivalis* has been classified into four genotypes (HG66, W83, W83v, and 381) based on the heterogenic nucleotide alignment (36). *P. gingivalis* capsules were also classified into six types (K1–K6) based on the K-antigen serotypes and this serotype variation was suggested to have an influence on bacterial virulence (31). Thus, we examined those phenotypic and genotypic variations among the present isolates (Table 3). Our results showed that eight of the nine experimental isolates showed negligible autoaggregation, whereas TDC285 and ATCC33277 became autoaggregated over time. All of the experimental isolates showed lower hydrophobicity than ATCC33277. Further, variations of the *kgp* genotype were observed but they did not seem to be related to pathogenic heterogeneity. Also, eight of the isolates were found to be encapsulated with the K4 or K5 serotype, while TDC285 was found to possess no capsule (K^- type), which was the same as ATCC33277.

Statistical analysis of pathogenic heterogeneity-related factors of *P. gingivalis* isolates with type II fimbriae

Quantitative host inflammatory parameters were statistically analyzed with respect to the relationship with bacterial factors (Table 4). Gingipain activities and efficiency for adhesion and invasion were shown to be positively related with induction of sialic acid concentration, with statistical significance shown regarding the relationship to secreted Kgp activity, as well as adhesion and invasion efficiency. In contrast, autoaggregation and hydrophobicity tended to be negatively related to inflammation. Other inflammatory parameters, such as spleen swelling and body weight loss, were also related to gingipain activities and the efficiencies of adhesion and invasion, with invasion efficiency shown to be significantly related.

Invasion efficiency-related factors of *P. gingivalis* isolates with type II fimbriae

Since the invasive efficiency of *P. gingivalis* was shown to be strongly related to bacterial virulence, the above bacterial factors were analyzed with respect to their relationship to invasion efficiency. As shown in Table 5, the proteolytic activities of Rgp and Kgp were positively related to

Table 3. Phenotypic and genotypic variations among *Porphyromonas gingivalis* isolates with type II fimbriae

Isolate	Autoaggregation ($-d_{abs}/d_t$)	Hydrophobicity (%)	*kgp* type	Capsular type
TDC60	0.001 ± 0.001	7.73 ± 0.19	HG66	K4
TDC117	0.004 ± 0.001	14.80 ± 3.03	HG66	K5
TDC275	0.002 ± 0.002	14.55 ± 3.27	HG66	K5
OMZ314	0.000 ± 0.001	13.85 ± 3.83	HG66, 381, W83	K4
TDC120	0.003 ± 0.003	63.01 ± 5.56	HG66	K4
TDC59	0.001 ± 0.001	8.14 ± 1.12	HG66	K4
TDC222	0.002 ± 0.002	27.14 ± 2.44	HG66	K5
TDC243	0.027 ± 0.008	9.45 ± 3.20	HG66, 381,W83v	K4
TDC285	0.003 ± 0.001	34.16 ± 3.49	HG66	K^-
ATCC33277	0.029 ± 0.004	76.09 ± 4.72	HG66, 381	K^-

adhesion/invasion and invasion, respectively, while secreted gingipain activity was significantly related to the invasive efficiency of *P. gingivalis*.

Discussion

In the present study, many of the type II fimbria isolates of *P. gingivalis* caused severe inflammation in the mice, others were less causative, as was the control strain ATCC33277. Thus, pathogenic heterogeneity was found to exist among *P. gingivalis* clones with type II fimbriae and our results also suggested that the heterogeneity was related to gingipain activities. Gingipains are secreted proteins that exist on bacterial cell surfaces, are associated with extracellular membrane vesicles, and have been found in culture supernatants (reviewed in ref. 23). Both Rgp and Kgp contain propeptide, catalytic (proteolytic), and adhesin domains, and the proteolytic actions of gingipains are widely known to be a major virulence factor of *P. gingivalis*. In addition to significant proteolytic activity to degrade host proteins, Rgp induces vascular permeability enhancement through activation of the kallikrein/kinin pathway and activates the blood coagulation system, which has been associated with the progression of inflammation in a mouse model (23). Kgp is the most potent fibrinogen/fibrin-degrading enzyme in human plasma and has been shown to be involved in bleeding tendency at infected sites in mice (23). Gingipains degrade CD14, the cellular receptor for bacterial lipopolysaccharide, thus inhibiting the activation of leukocytes through Toll-like receptors, thereby facilitating the survival of *P. gingivalis* (23). In addition, our previous *in vitro* findings showed that type II *P. gingivalis* efficiently invades epithelial cells and degrades focal adhesion components with Rgp, which causes cellular impairment during wound healing and tissue regeneration (40). Those findings support the notion that gingipain activities (especially those of secreted gingipains) are related to pathogenic heterogenicity among *P. gingivalis* isolates with type II fimbriae.

Pathogenic heterogeneity has been shown to be related to invasion efficiency. Epithelial and endothelial cells are spontaneously exposed to bacterial attacks, and prevent invasion into deeper tissues. This part of the innate host defense system is thought to limit the spread of invaded bacteria by maintaining an intact barrier (15). In previous studies, *P. gingivalis* was detected in the gingival tissues of periodontitis patients (43), and shown to invade human epithelial, endothelial, fibroblastic, and periodontal ligament cell lines *in vitro* (7, 33). This intracellular pathogen also degrades cellular signaling molecules and disables such cellular functions as adhesion, migration, and proliferation, which are crucial for the homeostatic, regenerative, and healing properties of periodontal tissues (21, 24, 26, 40). In addition, such localization allows the pathogen to penetrate deep into tissues (55). This adhesive/invasive ability seems to promote the ability of *P. gingivalis* to destroy host tissues and it is possible that the invasion efficiency of *P. gingivalis* is related to pathogenic heterogenicity.

Type II fimbriae have been shown to be the most adhesive and invasive among the six fimbrial types, and promote bacterial invasion of the host cells in a significantly greater manner than the other types. This prominent efficiency has been ascribed to the significantly higher affinity of type II fimbriae for $\alpha_5\beta_1$-integrin, as compared with other fimbrial types (26). However, the present study showed that invasive efficiency varied among type II clones, while gingipain activities, especially those of extracellular gingipains, were shown to be related to invasive efficiency. Gingipains are able to degrade various host proteins, but not those of *P. gingivalis* fimbriae, which were reported to exhibit a strong affinity to the arginine residues of the matrix proteins (28). Furthermore, arginine-containing peptide inhibited co-aggregation (cohesive interaction) of *P. gingivalis* with other plaque-forming bacteria (2). It was also shown that separate treatments of gingival fibroblasts and extracellular matrix proteins with purified Rgp enhanced their binding of fimbriae (28, 29). Thus, it is possible that Rgp exposes arginine residues as cryptic ligands for fimbriae, following degradation of cell surface proteins. Gingipain adhesion domains were also shown to promote bacterial adherence to epithelial cells (11), which can influence the invasive ability of *P. gingivalis*. It was also suggested that the catalytic and adhesive domains form complexes and bind to epithelial cells, and that

Table 4. Pathogenic heterogeneity-related factors of *Porphyromonas gingivalis* isolates with type II fimbriae

	Cell extract		Culture supernatant					
	Rgp	Kgp	Rgp	Kgp	Autoaggregation	Hydrophobicity	Adhesion/Invasion	Invasion
(i) Relationship with sialic acid								
r value	0.603	0.605	0.588	0.748	−0.422	−0.121	0.802	0.870
P value	0.065	0.064	0.074	0.010*	0.271	0.766	0.004 *	<0.001 *
(ii) Relationship with spleen swelling								
r value	0.342	0.432	0.376	0.580	−0.355	−0.117	0.590	0.768
P value	0.346	0.221	0.295	0.795	0.364	0.774	0.732	0.007 *
(iii) Relationship with body loss								
r value	−0.381	−0.473	−0.449	−0.660	0.181	0.322	−0.710	−0.796
P value	0.288	0.174	0.201	0.036 *	0.655	0.414	0.019*	0.004 *

*Significant relationship.

Table 5. Invasion efficiency-related factors of *Porphyromonas gingivalis* isolates with type II fimbnae

	Cell extract		Culture supernatant			
	Rgp	Kgp	Rgp	Kgp	Autoaggregation	Hydrophobicity
(i) Relationship with adhesion/invasion						
r value	0.438	0.457	0.620	0.781	−0.335	−0.154
P value	0.214	0.192	0.055	0.006	0.393	0.705
(ii) Relationship with invasion						
r value	0532	0.584	0.634	0.802	−0.409	0.023
P value	0.116	0.077	0.048	0.004	0.288	0.965

*Significant relationship.

gingipain proteolytic activities can mediate the detachment of bacteria and thus modulate adhesion (12). These findings support the possibility that gingipains are related to bacterial adhesion and invasion.

Autoaggregation of *P. gingivalis* is thought to be an important process in biofilm formation (27) and fimbriae are considered to be involved (19). In addition, *P. gingivalis* invades host cells through a cellular actin-mediated pathway (49) and efficient uptake of the bacteria by host cells is dependent on the size of the endocytic organisms (13). It is possible that the invasion of host cells by *P. gingivalis* is suppressed by autoaggregation and the negligible autoaggregation ability of type II fimbriae probably contributes to its invasion of host cells.

The *P. gingivalis* strains classified as K-antigen capsular types (K1–K6) were reported to be pathogenic clones in a mouse abscess model (32). Many type II isolates have been found to be encapsulated and K-typeable, whereas TDC285 and ATCC33277, both of which caused the lowest levels of inflammatory changes in the present study, are not. Encapsulation seems to be a virulence determinant. Surface hydrophobicity is thought to promote bacterial interactions with host proteins and phagocytosis by leukocytes (48), and the capsule of *P. gingivalis* lowers the hydrophobicity of the bacterial surface (8). Although the results were not statistically significant, bacterial hydrophobicity was negatively related to bacterial pathogenicity in the present mouse model. The low hydrophobicity of type II isolates may provide resistance to phagocytosis and promote its survival, resulting in extensive inflammation.

The present findings also suggest the possibility that clinical isolates with other types of fimbriae have pathogenic heterogeneity. Many strains of *P. gingivalis* were classified as either avirulent/non-invasive or virulent/invasive in mouse models (reviewed in ref. 14), and we previously analyzed the relationships between those virulence/invasiveness and the fimbrial types (41). Although fimbrial type was apparently shown to be a major virulence determinant of *P. gingivalis*, pathogenic heterogeneity was suggested among the clones sharing the same fimbrial type. The heterogeneity-related factors of *P. gingivalis* strains with other fimbria types are expected to be further examined. In addition, an orally-infected murine periodontitis model is also useful for future investigations.

The standard assays used for adhesive and invasive efficiencies of isolates are colony-forming unit counts and [^{3}H]thymidine counting (40) assays. In this study, we used membrane vesicle-conjugated MSB to assess the adhesive and invasive efficiencies of the isolates. We initially employed a standard adhesion assay with viable bacterial cells; however, bacterial cells of four of the isolates were found floating in the cell culture medium and scarcely interacted with epithelial cells layered on the bottom of the culture wells. Although it is unknown why these isolates tended to float in the medium, such a characteristic must be considered for adequate estimation of adhesion/invasive ability. A previous study that used viable cells of *P. gingivalis* clinical isolates with various *fimA* types found that most strains were able to adhere to and invade epithelial cells, though those properties were not homogeneous among strains belonging to the same fimbrial type (50), as some of those isolates might also have floated in that assay medium.

In summary, though type II fimbriae are a significant virulence factor of *P. gingivalis*, the present results suggest that pathogenic heterogeneity exists in the relationships with invasive and gingipain activities among *P. gingivalis* clones with type II fimbriae.

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