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Porphyromonas gingivalis enhances pneumococcal adhesion to human alveolar epithelial cells by increasing expression of host platelet-activating factor receptor

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Streptococcus pneumoniae **causes pneumonia by infecting the alveolar epithelium *via* binding to host receptors, such as the platelet-activating factor receptor (PAFR). Although chronic periodontitis has been identified as a pneumonia risk factor, how periodontopathic bacteria cause pneumonia is not known. We found that *S. pneumoniae* adhered to PAFR expressed on A549 human alveolar epithelial cells stimulated by *Porphyromonas gingivalis* culture supernatant, and this was abrogated by a PAFR-specific inhibitor. Among the major virulence factors of *P. gingivalis* [lipopolysaccharide (LPS), fimbriae and gingipains (Rgps and Kgp)], PAFR expression and pneumococcal adhesion were executed in an Rgp-dependent manner. LPS and fimbriae did not induce PAFR expression. Hence, our findings suggest that *P. gingivalis* enhances pneumococcal adhesion to human alveoli by inducing PAFR expression and that gingipains are responsible for this.**

Keywords: adhesion; alveoli; gingipain; lung; PAFR; platelet-activating factor receptor; pneumonia; *Porphyromonas gingivalis*; *Streptococcus pneumoniae*

Pneumonia, including community-acquired pneumonia and nosocomial pneumonia, is a pulmonary inflammation possibly caused by exogenous insults, such as microbes, and is a major health problem worldwide because of its high mortality rates, particularly in the elderly [1,2]. Human lower respiratory epithelia, such as the alveolar epithelium, become infected by exogenous respiratory bacteria, such as *Streptococcus pneumoniae*. Bacterial surface molecules known as phosphorylcholine, a structural mimic of platelet-activating factor, bind specifically to host receptors, such as platelet-activating factor receptor (PAFR), on the epithelial cells [3,4]. Bacterial binding to the epithelium subsequently causes bacterial infection that induces pro-inflammatory responses in the alveolar epithelium, thereby contributing to the development of bacterial pneumonia.

PAFR expressed on the pulmonary epithelium is inducible by stimulation with inhaled materials, such as acid, fossil-fuel-derived particulate matter, and cigarette smoke [3]. In addition, expression tends to increase in an age-dependent manner [5]. Furthermore, PAFR is increased by the infection of several viruses, such as rhinovirus and influenza virus, and usually causes a more severe secondary pneumococcal pneumonia [6,7]. Importantly, an increase in the amount of PAFR is linked to an increase in the level of pneumococcal adhesion because PAFR-deficient mice are resistant to pneumococcal pneumonia compared with their wild-type controls [8]. This strongly indicates that the

Abbreviations
BALF, bronchoalveolar lavage fluid; BHI, brain–heart infusion; CFU, colony-forming units; CSP, culture supernatant of *P. gingivalis*; DMEM, Dulbecco's modified Eagle's medium; Kgp, lysine-specific gingipain; LPS, lipopolysaccharide; MOI, multiplicity of infection; PAFR, platelet-activating factor receptor; PCR, polymerase chain reaction; Rgp, arginine-specific gingipain.

induction of PAFR on the alveolar epithelium is of great importance and directly leads to the onset of pneumococcal pneumonia.

Chronic periodontitis is an inflammatory and infectious condition and is a prevalent disease worldwide [9]. This illness causes destruction of the periodontium, including the periodontal bone, leading to loss of teeth when left untreated. Inflammatory responses triggered by the increased numbers of endogenous periodontopathic bacteria underlie this condition. *Porphyromonas gingivalis* is a highly pathogenic, Gram-negative and black-pigmented anaerobe that colonises periodontal pockets and becomes detectable in the saliva of patients as chronic periodontitis progresses. The bacterium harbours virulence factors, such as lipopolysaccharide (LPS), fimbriae and trypsin-like proteinases, known as gingipains, which are species-specific enzymes: Rgp that cleaves arginine residues and lysine-specific gingipain (Kgp) that cleaves lysine residues [10].

Accumulating evidence indicates that chronic periodontitis is a risk factor for several systemic diseases, such as preterm birth, diabetes and atherosclerosis [9]. In this regard, we have reported that the culture supernatant of *P. gingivalis* (CSP) possibly induces the reactivation of latently infected viruses, namely human immunodeficiency virus and Epstein–Barr virus [11,12]. Over the last two decades, chronic periodontitis has become a known risk factor for pneumonia, especially in the elderly [9]. In fact, an increase in the number of teeth with periodontal pockets in the elderly is associated with increased mortality from pneumonia [13]. Probing depth and clinical attachment level, both of which represent the degree of periodontal bone loss, correlate with clinical indices of pneumonia. Furthermore, periodontal intervention, such as oral hygiene instruction, reduces the occurrence of pneumonia, even among high-risk individuals [14]. In addition, the elderly are particularly susceptible to aspiration due to reduced laryngopharyngeal sensitivity [15]. Indeed, *P. gingivalis* is isolated from bronchoalveolar lavage fluid (BALF) or sputum from patients with pneumonia [16,17]. These observations have led to the hypothesis that *P. gingivalis* present in the saliva is aspirated into the lower respiratory tract, thereby positioning it to contribute to the development of pneumonia. Despite its importance, a causal relationship between *P. gingivalis* and pneumonia remains unexamined.

Many studies have used CSP to examine the virulence factors of *P. gingivalis*. Indeed, with human periodontal and immune cells, CSP stimulates pro-inflammatory characteristics, such as induction of inflammation-related receptor expression and pro-inflammatory cytokine secretion [18–21], all of which contribute to the virulence of this bacterium. However, whether CSP also stimulates pro-inflammatory characteristics with the alveolar epithelium has not been reported. We were, therefore, interested in the capability of CSP to induce inflammation-related receptor expression. Herein, we report on CSP-induced PAFR expression on human alveolar epithelial cells owing to gingipains in CSP, which consequently enhanced the adhesion of *S. pneumoniae* to the epithelial cells.

Our results uncovered a novel mechanism by which *P. gingivalis* induces pneumococcal adhesion to lung-derived epithelial cells and suggest that this interaction may contribute to an increased risk of pneumococcal pneumonia in the context of periodontal disease.

Materials and methods

Reagents

The PAFR-specific inhibitor WEB2086 was purchased from Focus Biomolecules (Plymouth Meeting, PA, USA). A stock solution of WEB2086 was prepared in DMSO. *P. gingivalis* LPS was obtained from InvivoGen (San Diego, CA, USA). Fimbriae were kindly provided by Y. Hasegawa (Aichi Gakuin University, Aichi, Japan) [22].

Cell culture

The human alveolar epithelial cell line A549 was maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) with 10% FBS, penicillin 100 $U \cdot mL^{-1}$ and streptomycin 100 $\mu g \cdot mL^{-1}$ at 37 °C in a 5% CO_2 atmosphere.

Bacterial strains

Porphyromonas gingivalis strain ATCC 33277 was grown in brain–heart infusion (BHI) broth (Becton, Dickinson and Company, Sparks, MD, USA) supplemented with 5 $\mu g \cdot mL^{-1}$ hemin and 0.4 $\mu g \cdot mL^{-1}$ menadione in an anaerobic system (5% CO_2, 10% H_2 and 85% N_2 at 37 °C using a model 1024 anaerobic chamber; Forma Scientific, Marietta, OH, USA) for 48 h. The supernatant was collected by centrifugation at 10 000 ***g*** at 4 °C for 20 min to remove the bacteria and filter-sterilised through a 0.22-μm pore size membrane filter. The supernatant pH values ranged 6.8–7.0. ATCC 33277-derived mutant strains KDP129 (*Δkgp*), KDP133 (*ΔrgpA ΔrgpB*) and KDP136 (*Δkgp ΔrgpA ΔrgpB*) were kindly provided by K. Nakayama (Nagasaki University, Nagasaki, Japan) [23,24]. KDP129, KDP133, KDP136 or wild-type strain ATCC 33277 were cultured to the late log phase, and supernatants were collected. *S. pneumoniae* ATCC6303 (serotype 3) was grown in BHI broth at 37 °C for 24 h until the late exponential phase.

RT-qPCR assay

Cells were stimulated with medium containing *P. gingivalis* culture supernatant (100 $\mu L \cdot mL^{-1}$), LPS (0.1 or 1 $\mu g \cdot mL^{-1}$) or fimbriae (0.1 or 1 $\mu g \cdot mL^{-1}$) and incubated at 37 °C for 12 or 24 h. Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The synthesis of cDNA from total RNA was performed with PrimeScript™ RT Master Mix (Takara Bio, Shiga, Japan). Transcript levels of PAFR were assessed using quantitative real-time polymerase chain reaction (PCR) (Takara Bio) that was performed by the SYBR Green method. Primer sequences used for the amplification of each gene were as follows: PAFR, forward 5′-GTGG GCTGGACTTGGCTGAT-3′ and reverse 5′-CCTGGTCC CTCAGCAGGAAA-3′, and GAPDH, forward 5′-ACCA GCCCCAGCAAGAGCACAAG-3′ and reverse 5′-TTCAA GGGGTCTACATGGCAACTG-3′. PCR assays were performed using a TP-800 Thermal Cycler Dice Real-Time System (Takara Bio). These experiments were performed in triplicates. Calculated gene expression levels were normalised to GAPDH mRNA levels.

Western blot analysis

After treating with CSP for 24 h, cells were washed by PBS and then lysed with RIPA buffer. The proteins were separated by SDS/PAGE and transferred to PVDF membranes. The membranes were blocked with 2% BSA and incubated with primary antibodies overnight at 4 °C. Primary antibodies included anti-PAFR antibody (Cayman Chemical, Ann Arbor, MI, USA) or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were washed with Tris-buffered saline containing Tween 20 and incubated with secondary antibody for 1 h at room temperature. After washing, protein bands were detected using the ChemiDoc XRS System (Bio-Rad, Hercules, CA, USA).

Immunofluorescence

Streptococcus pneumoniae suspension was prepared in 0.1 M NaCl-50 mM sodium carbonate buffer (pH 9.5) at 1×10^8 colony-forming units (CFU)/mL. FITC-I (Dojindo Laboratories, Kumamoto, Japan) was added at a concentration of 50 $mg \cdot mL^{-1}$, and the mixture was incubated at 4 °C for 1 h. The cells were washed three times with PBS. A549 cells grown on cover slides pretreated with poly-L-lysine (Matsunami Glass, Osaka, Japan) were treated with CSP for 24 h, and thereafter, the cells were incubated with FITC-labelled *S. pneumoniae* at a multiplicity of infection (MOI) of 10 CFU/cell for 1 h. Slides were washed with PBS and then fixed with 4% paraformaldehyde (Wako Pure Chemical Industries, Osaka, Japan) for 20 min at room temperature. The slides were blocked with 2% BSA for 1 h and incubated with anti-PAFR monoclonal antibody (Cayman Chemical) in blocking buffer overnight at 4 °C. After thorough rinsing with PBS, an Alexa Fluor 555 anti-mouse secondary antibody (Thermo Fisher Scientific, Waltham, MA, USA) in PBS containing 2% BSA and Hoechst 33342 (Thermo Fisher Scientific). Cover slides were mounted on glass slides with ProLong Gold Antifade Reagent (Thermo Fisher Scientific) and examined using a fluorescence microscope (IX-FLA; Olympus, Tokyo, Japan). The images obtained were subsequently analysed using InStudio (Pixera, Santa Clara, CA, USA).

Luciferase assay

A549 cells were seeded in 24-well plates and were transfected with a PAFR promoter-reporter plasmid p.98Luc (kindly provided by J. Staňková, Faculté de Médecine et Sciences de la Santé Université de Sherbrooke Québec, Canada) [25], using Lipofectamine 2000 Transfection Regent (Invitrogen, Gaithersburg, MD, USA). Cells were then treated with CSP and subjected to the Luciferase Assay System (Promega, Madison, WI, USA).

Adhesion assay

An adhesion assay was performed using a standard bacterial adhesion assay [26]. Briefly, A549 cells were cultured in confluent in 24-well plates and treated with *P. gingivalis* culture supernatants for 24 h. The plate was washed twice with DMEM (penicillin-free) and then incubated with *S. pneumoniae* suspension at an MOI of 10 CFU/cell for 1 h. The plate was washed three times, and A549 cells were removed from the plate with trypsin EDTA and lysed with distilled water for 10 min. Serial dilutions of lysates were plated on BHI agar plates to determine the number of CFU of the adhered bacterial cells.

Statistical analysis

All experiments were performed at least three times, and mean values ± SD were calculated. Statistical analysis was performed using either one-way ANOVA with Tukey's *post hoc* analysis or Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

Results

CSP as a stimulant to induce PAFR expression in A549 human alveolar epithelial cells

To investigate whether CSP is capable of upregulating PAFR transcription in the A549 alveolar epithelial cells, we quantified the mRNA expression of PAFR using RT-qPCR. A significant upregulation of PAFR mRNA expression was observed in the presence of CSP (Fig. 1A). Incidentally, CSP also increased PAFR

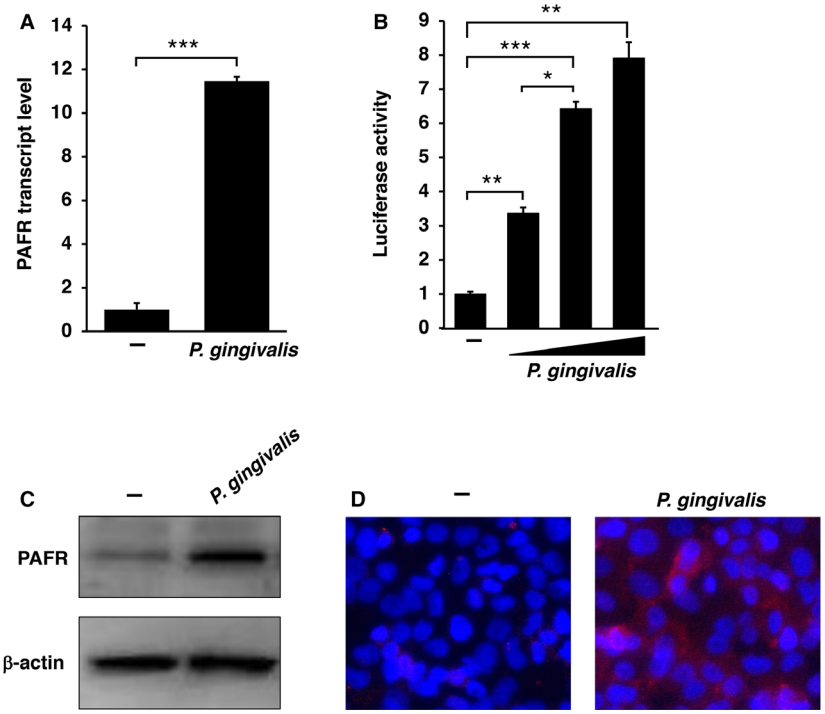

Fig. 1. Culture supernatant of *P. gingivalis* (CSP) upregulates PAFR expression on A549 cells. (A) A549 cells were treated with bacterial growth medium (control) or CSP (100 μL·mL^{-1}) for 12 h. PAFR transcript level was determined by RT-qPCR. The values are presented as mean ± SD; $n = 3$ (***, $P < 0.001$). (B) A549 cells were transfected with the luciferase reporter construct of the PAFR promoter (p98 Luc) for 24 h. Thereafter, the cells were treated with bacterial growth medium (control) or CSP (12.5, 25, 50 μL·mL^{-1}) for 24 h, and luciferase activity was determined in cell lysates. The values are presented as mean ± SD; $n = 3$ (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). (C) A549 cells were treated with bacterial growth medium (control) or CSP (100 μL·mL^{-1}) for 24 h. PAFR protein expression was detected by western blotting of whole cell lysates. (D) A549 cells were treated with bacterial growth medium (control) or CSP (100 μL·mL^{-1}) for 24 h. PAFR protein expressed on A549 cells was detected by immunofluorescence analysis. Depicted is the merged image of PAFR (red) and nucleus (blue). Data are the results of three independent experiments. Images shown are representative examples of one of the three experiments. Statistical significance was evaluated by Student's *t*-test (A) or by one-way ANOVA with Tukey's *post hoc* analysis (B).

mRNA expression in the human bronchial epithelial cell line Calu-3 (data not shown). To test whether CSP activates the PAFR promoter in A549 cells, we transfected the luciferase reporter construct of the PAFR promoter to the cells and performed a luciferase assay. Luciferase was activated in the presence of CSP in a dose-dependent manner (Fig. 1B). In addition, PAFR protein expression was confirmed by western blotting (Fig. 1C) and immunofluorescence staining (Fig. 1D) of A549 cells was treated with CSP. PAFR protein was distributed on the surface of the A549 cells (Fig. 1D). Therefore, CSP stimulates PAFR mRNA and protein expression on A549 cells.

Adherence of *S. pneumoniae* to human alveolar epithelial cells stimulated by CSP

Because CSP induced expression of PAFR on A549 cells, we were interested in whether the expressed PAFR functioned as a receptor for *S. pneumoniae* adhesion. Pretreatment with CSP induced an increase of ~ 50% in the number of *S. pneumoniae* that adhered to A549 cells (Fig. 2A,B). Then, we examined whether the observed increase in the pneumococcal adhesion to the epithelial cells was caused by the induced expression of PAFR by using a specific PAFR inhibitor, WEB2086. Both PAF and WEB2086 dock into the same deep cavity on the extracellular surface of the PAFR molecule as reported in an in silico analysis [27]. CSP-induced pneumococcal adhesion was totally abrogated in the presence of WEB2086 (Fig. 2C,D), indicating that this CSP-induced pneumococcal adhesion to A549 cells was dependent on PAFR.

Contribution of Arg-gingipain in CSP to PAFR-dependent adherence of *S. pneumoniae* to human alveolar epithelial cells

To seek the major components of CSP responsible for the observed increase in pneumococcal adhesion,

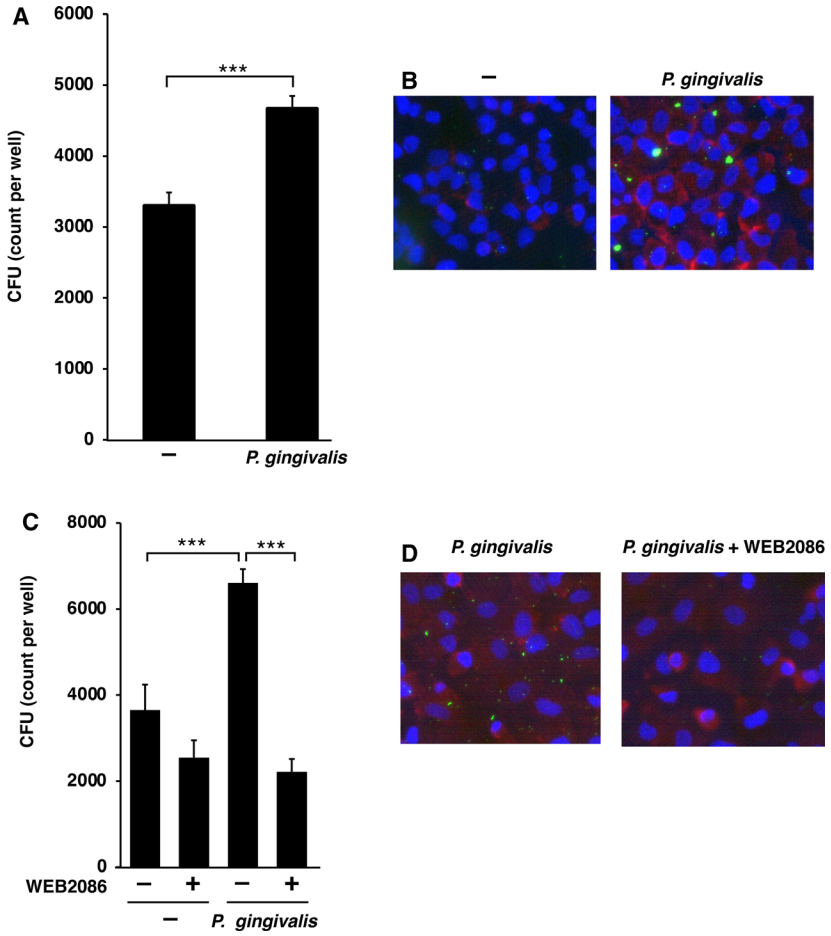

Fig. 2. CSP promotes adhesion of *S. pneumoniae* to A549 cells, which is abrogated by PAFR-specific inhibition. A549 cells were treated with bacterial growth medium (control) or CSP (100 μL·mL^{-1}) for 24 h. Thereafter, half of the cells were inoculated with *S. pneumoniae* at a MOI of 10 CFU/cell for 1 h. The other half of the cells were treated with WEB2086 (10 μM) for 1 h and then inoculated with *S. pneumoniae* as described above. (A) The adhesion of *S. pneumoniae* to A549 cells was determined by adhesion assays. The values are presented as mean ± SD; $n = 6$ (***, $P < 0.001$). (B) Adhesion of *S. pneumoniae* to A549 cells was visualised by immunofluorescence analysis. Depicted is the merged image of PAFR (red), nucleus (blue) and *S. pneumoniae* (green). (C, D) The adhesion of *S. pneumoniae* to A549 cells pretreated with DMSO or WEB2086 (10 μM) was determined and visualised in the same manner as described in (A) and (B), respectively. Data are the results of three independent experiments. Images shown are a representation of one of the three independent experiments. Statistical significance was evaluated by Student's *t*-test (A) or by one-way ANOVA with Tukey's *post hoc* analysis (C).

P. gingivalis-derived LPS and fimbriae, both of which appear in CSP [28], were applied to A549 cells. Unexpectedly, neither LPS nor fimbriae induced PAFR transcription in these cells (Fig. 3A). We next examined the effects of the third major CSP component: gingipains designated as HRgpA (RgpA), RgpB and Kgp. We used CSPs of several isogenic gingipain-mutant strains (offspring of ATCC 33277): KDP129 (deficient in *kgp*) [23], KDP133 (deficient in *rgpA* and *rgpB*) and KDP136 (deficient in *kgp*, *rgpA* and *rgpB*) [24]. Neither the CSP of KDP133 nor that of KDP136 induced PAFR transcription in A549 cells (Fig. 3B). By contrast, the CSP of KDP129 upregulated PAFR transcription to the same extent as its parent strain ATCC 33277 ($P > 0.05$). These data indicate that the Rgp as a component of CSP was predominantly responsible for the observed induction of PAFR. Moreover, neither the CSP of KDP133 nor that of KDP136 induced the pneumococcal adhesion (Fig. 3C), indicating that Rgp was necessary for the induction of pneumococcal adhesion *via* the precedent induction of PAFR on the alveolar epithelium.

Discussion

Pneumonia is an acute and inflammatory infectious condition developed in the alveoli and bronchioles of the lung and includes community-acquired pneumonia and healthcare-associated pneumonia. Pneumonia is initiated upon infection in respiratory epithelium by exogenous respiratory bacteria, such as *S. pneumoniae*, non-typeable *Haemophilus influenzae* and *Pseudomonas aeruginosa* [29]. These bacteria express an extracellular molecule that functions as a ligand (adhesin) that binds to a human respiratory epithelial receptor, and with this interaction, they infect the alveolar epithelium, where the bacterial adhesin is phosphorylcholine and the epithelial receptor is PAFR. Using PAFR-deficient mice, van den Poll and his coworkers have revealed that among these bacteria, *S. pneumoniae* alone further exploits PAFR for progressing experimental pneumonia [7,8,30]. Because we were interested in the adhesion of the bacteria that worsens pneumonia, we examined *S. pneumoniae* in the present study. Our findings reveal several

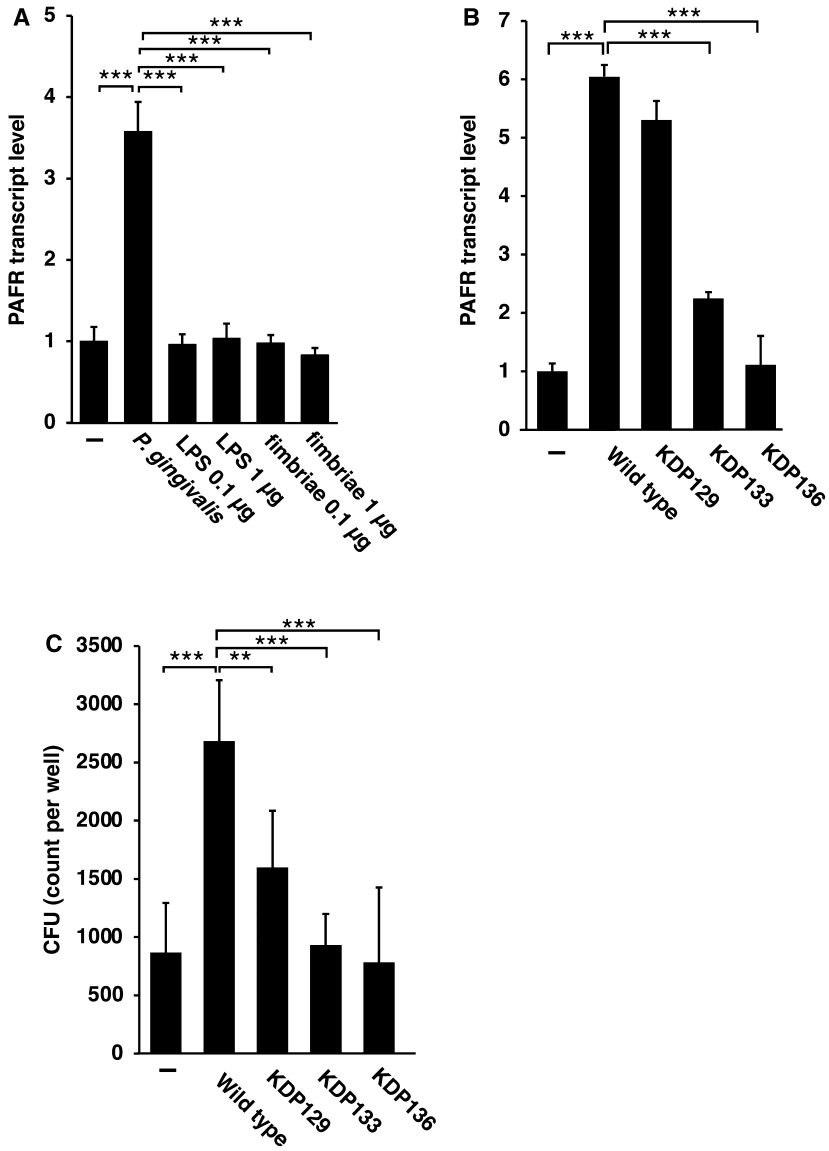

Fig. 3. Effects of major virulence factors in CSP on PAFR expression and adhesion of *S. pneumoniae* to A549 cells. (A) A549 cells were treated with bacterial growth medium (control) or CSP (100 μL·mL^{-1}), LPS (0.1 or 1 μg·mL^{-1}), and fimbriae (0.1 or 1 μg·mL^{-1}) for 24 h. PAFR transcript level was determined by RT-qPCR. The values are presented as mean ± SD; $n = 3$ (***, $P < 0.001$). (B) A549 cells were treated with bacterial growth medium (control) or CSP obtained from wild-type bacterium or those with gingipain-deficient mutants (KDP129; *Δkgp*, KDP 133; *ΔrgpA ΔrgpB* or KDP 136; *Δkgp ΔrgpA ΔrgpB*) (100 μL·mL^{-1}) for 12 h. Thereafter, PAFR transcript level was determined by RT-qPCR. The values are presented as mean ± SD; $n = 3$ (***, $P < 0.001$). (C) A549 cells were treated with bacterial growth medium (control) or CSP or CSPs obtained with gingipain-deficient mutants for 24 h and then incubated with *S. pneumoniae* at a MOI of 10 CFU/cell for 1 h. The adhesion of *S. pneumoniae* was determined by adhesion assay. The values are presented as mean ± SD; $n = 6$ (**, $P < 0.01$; ***, $P < 0.001$). Data are the results of three independent experiments. Statistical significance was evaluated using one-way ANOVA with Tukey's *post hoc* analysis (A–C).

phenomena for the first time: CSP induced PAFR expression on A549 human alveolar epithelial cells, which consequently promoted pneumococcal adhesion, which occurred due to *P. gingivalis* gingipains.

Chronic periodontitis is a risk factor for pneumonia, presumably due to the aspiration of saliva contaminated with periodontopathic bacteria and their products into the lower respiratory tract. In addition, approximately half of the healthy adults experience salivary aspiration during sleep [31], but the risk of aspiration is particularly increased in the elderly because of reduced laryngopharyngeal sensitivity resulting in impairment of the cough and swallowing reflexes [32,33]. This may allow saliva contaminated with *P. gingivalis* and its products to be continually aspirated into the lower respiratory tract. Indeed, *P. gingivalis*, which is not an indigenous constituent of the lower respiratory microflora, has been isolated from sputum and BALF of patients with pneumonia [16,17]. In addition, although a concept that aspiration of oral pathogens such as *P. gingivalis* into the lung and periodontal disease-associated enzymes, such as gingipains, in the saliva may modify mucosal surfaces to promote adhesion and infection of respiratory pathogens, such as *H. influenzae*, has been postulated [29], the underlying mechanisms of this consequence have not been examined to date. We therefore tested whether CSP induced PAFR expression on human alveolar epithelial cells and whether this promoted adhesion of *S. pneumoniae* to the epithelial cells.

Incidentally, some *S. pneumoniae* cells were observed to adhere in a small cluster-forming manner; however, this never hindered assessing the level of bacterial adhesion because the measurement was conducted on the basis of the CFU count. Our present study hence shows the capability of CSP to induce PAFR expression, resulting in the promotion of pneumococcal adhesion. Importantly, pneumococcal adhesion was completely abrogated in the presence of specific inhibitor of PAFR. In addition, keratin 10, polymeric immunoglobulin receptor and laminin receptor are known as host receptors for pneumococcal adhesion [4]. These receptors do not appear to be involved in the increase in pneumococcal adhesion ascribable to CSP because the inhibitor used completely restored pneumococcal adhesion to control levels. Our observations herein together with previous findings suggest that *P. gingivalis* likely contributes to the development of pneumococcal pneumonia through the induction of PAFR expression on the alveolar epithelium.

We investigated three major components of CSP: LPS, fimbriae and gingipains. These materials are known bacterial products exclusively released from bacterial cells into CSP during a cultivation period leading to a late logarithmic phase, such as 48 h, at which the bacterial autolysis has not started yet [34], and have been studied for the past decades as the main virulence factors of *P. gingivalis* [35]. LPS derived from *P. gingivalis* causes a highly innate immune response and induces secretion of pro-inflammatory cytokines such as interleukin (IL)-1, IL-6, IL-8 and tumour necrosis factor (TNF)-α from host cells [10]. Fimbriae are a pivotal factor for adherence and invasion of host cells, and they also induce IL-1, IL-6 and TNF-α [10]. However, neither LPS nor fimbriae induced PAFR mRNA expression in A549 cells even at quite high concentrations, such as 1 $\mu g \cdot mL^{-1}$. We therefore examined gingipains as the remaining possible stimulant. Gingipains comprise more than 85% of whole proteases secreted by *P. gingivalis* [36]. Although they are recognised as hydrolytic enzymes, they also induce pro-inflammatory cytokine secretion and receptor expression [10,37]. Analyses of the gingipains revealed that the CSP of KDP136 (*rgpA rgpB kgp* triple mutant) neither induced PAFR mRNA nor promoted the pneumococcal adhesion to A549 cells. Both were ascribable predominantly to deficiency of Rgps but not to that of Kgp. Our findings were consistent with those reported by Benedyk et al. obtained from experimental aspiration pneumonia in mice induced by intratracheal inoculation of bacterial suspensions of a *P. gingivalis* strain or its gingipain-null mutants, which have concluded that pneumonia is ascribable to Rgps but not to Kgp [38]. Interestingly, those authors have additionally discussed that pneumonia is developed by an unknown function of the haemagglutinin–adhesin domain of RgpA, which promotes platelet infiltration in the lung and exacerbates inflammation and contributes to tissue necrosis, abscess formation and death in *P. gingivalis*-infected mice [38]. Indeed, neither heat inactivation of CSPs nor incubation with specific inhibitors for gingipains fully suppressed PAFR transcription in our study (data not shown). At this moment, between the haemagglutinin–adhesin domain and the catalytic domain, we cannot tell which one has more impact on inducing the observed upregulation. Therefore, this should be confirmed by additional studies utilising some molecular scientific methods, such as restoration of gingipain genes to the mutants and/or recombination of each gingipain domain gene. In addition, as PAFR expression is regulated by NF-κB and hypoxia-inducible factor-1α transcription factors [39–41], whether both or either signalling pathways are associated with the observed upregulation should be investigated. However, these issues remain outside the scope of the present study.

Chronic periodontitis and pneumonia are spreading worldwide. Although periodontitis has recently been identified as a risk factor for pneumonia, any causal relationship remains unclear. We have therefore aimed to delineate a putative mechanism by which *P. gingivalis* as one of the most periodontopathic bacteria that is aspirated into the lower respiratory tract may promote pneumococcal adhesion to human alveolar epithelia *via* the induction of PAFR as a pneumococcal receptor in an Rgp-dependent manner. Because periodontal care is effective in reducing or preventing pneumonia [42], it is therefore noted even at present that aspirated *P. gingivalis* should be identified as a hitherto unnoticed and more specific risk factor for the development of pneumococcal pneumonia.

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Data Accessibility

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author contributions

NK and MH designed the experiments, analysed the data and drafted the manuscript. MT and HT contributed to the conception of the study and performed experiments. KI was responsible for the study concept and design, analysis of results and manuscript writing. All authors read and approved the final manuscript.

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