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Porphyromonas gingivalis fimbriae-dependent interleukin-6 autocrine regulation by increase of gp130 in endothelial cells

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Background and Objective: Local persistent infection by *Porphyromonas gingivalis* leads to inflammatory systemic diseases, such as atherosclerosis. We have reported previously that avirulent *P. gingivalis* fimbriae-dependent invasion into endothelial cells might be involved in progression of atherosclerosis. Although interleukin-6 (IL-6) regulates progression of atherosclerosis, little is known about the relationship of *P. gingivalis* fimbriae-dependent invasion to IL-6 regulation in endothelial cells.

Material and Methods: We examined the secretion of IL-6 and the expression of the IL-6 signal transducer gp130 in human umbilical vein endothelial cells (HUVEC) infected with the wild-type FDC381 strain of *P. gingivalis* and a fimbriae-deficient mutant (*fimA*) by enzyme-linked immunosorbent assay, quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) and flow cytometry (fluorescence-activated cell sorting, FACS) analysis.

Results: Coculture of HUVEC with *P. gingivalis* resulted in increase of IL-6 secretion at 24 h postinfection. Interestingly, the increase was inhibited significantly in HUVEC infected with the *P. gingivalis fimA* mutant. In addition, the increase of IL-6 secretion induced by *P. gingivalis* infection was significantly impaired by the meiosis specific kinase 1 inhibitor, PD98059, or the nuclear factor κB inhibitor, Bay11-7082. Furthermore, we demonstrated that gp130 expression increased with *P. gingivalis* infection. Importantly, gp130 expression was significantly impaired by *P gingivalis fimA* mutant infection compared with wild-type *P. gingivalis* infection, as assessed by both quantitative RT-PCR and FACS analysis.

Conclusion: Our findings indicate that *P. gingivalis* fimbriae are important factors in the autocrine regulation of IL-6, by increasing gp130 in endothelial cells.

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Porphyromonas gingivalis is an oral pathogen that causes a chronic local inflammatory disease, periodontitis, resulting in the destruction of the periodontal ligament and alveolar bone (1). The bacterium possesses a broad array of virulence factors, including lipopolysaccharide (LPS), haemagglutinins, proteases and two types of fimbriae (41 kDa major fimbria and 67 kDa minor fimbria; 2). Recent reports in periodontal medicine suggest a definite relationship between persistent low-grade periodontal infections by *P. gingivalis* and certain systemic conditions,

including atherosclerosis and cardiovascular diseases (3–6).

Porphyromonas gingivalis fimbriae are critical factors that promote the bacterial invasion to the targeted sites (3,7). Thus, initial binding of *P. gingivalis* to host cells is mediated primarily via the major *P. gingivalis* fimbriae (3). *Porphyromonas gingivalis* fimbriae have been demonstrated to bind to various host cells, including human epithelial cells, erythrocytes and gingival fibroblasts (8–10). In common classifications, 41 kDa fimbriae (*fimA*) are classified into six *fimA* genotypes, and analyses of virulence for periodontitis have revealed both virulent and avirulent clones (9,10). The FDC381 strain of *P. gingivalis* has been classified as an virulent *fimA* genotype (3,4). We have reported previously that invasive strains of *P gingivalis* FDC381, but not a non-invasive *fimA* mutant, stimulate the expression of several cell adhesion molecules, such as intercellular adhesion molecule 1, vascular cell adhesion molecule 1, and P-/E-selectin on the cell surface of human umbilical vein endothelial cells (HUVEC; 3). In addition, infection with *P. gingivalis* can modulate the expression of chemokines, such as interleukin-8 (IL-8), in HUVEC through fimbriae-mediated mechanisms (4). Our findings have suggested that invasive *P. gingivalis* plays an important role in fimbriae-dependent induction of inflammatory molecules in endothelial cells.

Interleukin-6 is a pro-inflammatory cytokine that elicits a wide variety of biological activities in various cell types (11). Interleukin-6 signals are initiated by the phosphorylation of gp130, a common receptor for the IL-6 family, such as IL-11, leukaemia inhibitory factor (12). Increased levels of IL-6, C-reactive protein (CRP) and serum amyloid A correlate with prognosis in patients with the risk of coronary heart disease (CHD; 13). Recently, several large, prospective epidemiological studies have shown that plasma levels of IL-6 are strong predictors of future cardiovascular events in patients with a history of CHD (14). Furthermore, several *in vitro* experiments have shown that bacterial products of *P. gingivalis*, such as LPS and gingipains, promote IL-6 production in gingival fibroblasts, epithelial cells and KB (oral squamous cell carcinoma) cells (15).

Atherosclerosis, formerly considered a lipid storage disease, in fact involves an ongoing inflammatory response. Importantly, *P. gingivalis* has been identified in diseased human atherosclerotic tissues (16) and has been shown to increase the mean area and extent of atherosclerotic lesions in apolipoprotein-E knockout ($ApoE^{-/-}$) mice (5). Furthermore, it has been generally accepted that IL-6 is one of the inflammatory mediators reflected by an enhanced production of fibrinogen and CRP in liver and strongly affects the inflammatory process involved in the development of atherosclerosis through the stimulation of acute phase protein synthesis.

Since IL-6 plays a central role in the pathophysiology of cardiovascular disease, *P. gingivalis* infection might increase the IL-6-mediated inflammation through a fimbriae-dependent mechanism in endothelial cells. In the present study, therefore, we investigated: (1) the effects of *P. gingivalis* on both IL-6 and gp130 expression in endothelial cells; and (2) the effects of *P. gingivalis* fimbriae on IL-6/gp130 expression in endothelial cells by either invasive or non-invasive *P. gingivalis* strains. Recently, many researchers have reported the concept of periodontal medicine, interaction of local periodontal persistent infection and certain systemic diseases based on epidemiological studies or *in vitro* experiments (17,18). We believe that our *in vitro* models of persistent and low-grade infection targeting avirulent *P. gingivalis* fimbriae will contribute to the development of periodontal medicine.

Material and methods

Bacterial strains and growth conditions

Porphyromonas gingivalis wild-type (wt) strain FDC381 and the isogenic *fimA* mutant were gifts from Dr CA Genco at Boston University School of Medicine, Boston, MA, USA. The bacteria were routinely maintained as described previously (3–6). For all experiments, bacterial cells were incubated under anaerobic conditions.

Cell culture and infection with *P. gingivalis*

Human umbilical vein endothelial cells (HUVEC; Cascade Biologics Inc., Portland, OR, USA) were cultured in Media-200 supplemented with low-serum-growth supplement (20 μL/mL; Cascade Biologics, Inc.) at 37°C in 5% CO_2 in oxygen tissue culture flasks. For infection studies, cells were passed into six-well plates with 0.25% trypsin (Cascade Biologics, Inc.) and 0.02% EDTA (Boehringer Mannheim GmbH, Mannheim, Germany). Confluent cells were used in all experiments. The HUVEC were plated at a concentration of 10^5–10^6 cells/mL, as determined by cell counting with a haemocytometer (Hausser Scientific, Horsham, PA, USA). The multiplicity of infection (MOI) was calculated based on the number of cells per well at confluence. *Porphyromonas gingivalis* FDC381 (wt) and the isogenic *fimA* mutant grown to an optical density of 1.0 were centrifuged, washed with phosphate-buffered saline (PBS) and resuspended in HUVEC growth media at a final concentration of 10^8 cells/mL as described previously (3). Bacterial suspensions (1 mL) were added to confluent HUVEC at a MOI of 100:1 and incubated at 37°C in 5% CO_2 in oxygen for the indicated times.

After the total incubation period reached indicated times, *P. gingivalis*-infected HUVEC were collected and total RNA was isolated and processed as described by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Culture supernatants were also collected for enzyme-linked immunosorbent assay (ELISA). In addition, cells were collected either for Western blot analysis or for flow cytometry (fluorescence-activated cell sorting, FACS) analysis as described below. Control cultures were incubated with medium alone. To examine the effect of the extracellular regulated kinase (ERK) 1/2 pathway on *P. gingivalis* fimbriae-mediated IL-6 secretion, 10 μM PD98059 (Tocris Cookson Inc., Ellisville, MO, USA), a meiosis-specific

kinase 1 (MEK1) inhibitor, was added 60 min before infection. To examine the effect of the nuclear factor κB (NF-κB) pathway on *P. gingivalis* fimbriae-mediated IL-6 secretion, 2 μM Bay11-7082 (Tocris Cookson Inc.), a NF-κB inhibitor, was added 60 min before infection. These inhibitors were added throughout culture. All assays were performed in triplicate and repeated at least three times.

Immunoassay for IL-6

After *P. gingivalis*-infected HUVEC were cultured in the indicated conditions, the culture supernatants were collected and stocked at −80°C until use. The amount of IL-6 was measured using sandwich ELISA kits with antibody specific for human IL-6 (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Semi-quantitative RT-PCR and quantitative RT-PCR (qRT-PCR)

Total RNA was isolated from *P. gingivalis*-infected HUVEC using a RNeasy column (Qiagen, Valencia, CA, USA) and treated with RNase-free DNase (Qiagen) to remove contaminating genomic DNA according to the manufacturer's instructions. Using DNase-treated total RNA (2 μg), a first strand cDNA was synthesized with SuperScript II Preamplification System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Polymerase chain reactions were carried out using Platinum *Taq* (Invitrogen). Gene-specific primers were designed according to the sequences published in Genbank. The sets of PCR primers used were as follows: gp130, forward 5′-CCACCCGATCTTCATTCACT-3′ and reverse 5′-TAGTCCATTCCACC CAAAGC-3′; and GAPDH, forward 5′-NACCACAGTCCATGCCATCAC -3′ and reverse 5′-TCCACCACCCTG TTGCTGTA-3′. Next, a LightCycler® thermocycler was used to conduct real-time PCR (Roche Molecular Biochemicals, Mannheim, Germany). Assays were carried out in LightCycler® capillaries in a 20 μL reaction volume. Reaction reagents were purchased in a preformatted kit (LightCycler FastStart DNA Master SYBR Green I; Roche Diagnostics GmbH, Roche Molecular Biochemicals, Mannheim, Germany) containing ×10 concentrations of Taq DNA polymerase, deoxynucleoside triphosphates (dNTPs; with dUTP instead of dTTP), 10 mM $MgCl_2$, SYBR Green I and reaction buffer. The following concentrations proved optimal: forward primer, 0.5 μM; reverse primer, 0.5 μM; Taqman probe, 0.1 M; and $MgCl_2$, 5.0 mM. Each genomic equivalent of positive-control DNA was added in a 2 μL volume to 18 μL of master mix. No-template controls were prepared by adding a 2 μL volume of PCR-grade, sterile H_2O to 18 μL of master SYBR Green I mix. Thermocycling conditions were optimized to one cycle of denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 1 min and annealing of primers at 60°C for 1 min, with a single fluorescence acquisition step at the amplicon extension at 72°C for 10 min. A cycle of melting curve analysis of PCR product was then performed to confirm PCR accuracy with its primers. The gp130 mRNA fluorescence intensity was neutralized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with the in-built Roche LightCycler® software version 4.

Western blot analysis

At indicated times, cells were washed with PBS (pH = 7.3, Gibco, Invitrogen Co., NY, USA) and lysed in lysis buffer (20 mM Tris-HCl, pH 7.9, 137 mM NaCl, 2 mM phenylmethylsulphonic fluoride, 10 mM NaF, 5 mM EDTA, 5 mM dithiothreitol (DTT), 1 mM EGTA, 10% glycerol, 1 mM sodium pyrophosphate, 0.1 mM β-glycerophosphate, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 0.1% Triton X-100; Boehringer Mannheim GmbH, Mannheim, Germany). The protein concentrations of cell extracts were measured by Protein assay kit (Bio-Rad, Hercules, CA, USA). All samples were stored at −80°C until use. Aliquots from cell lysates (10 μg) were separated in a denaturing polyacrylamide gel and transferred to a poly (vinylidene fluoride) (PVDF) membrane (PerkinElmer Life Sciences, Inc., Boston, MA, USA). Membranes were blocked for 1 h at room temperature in Tris-buffered saline containing 0.1% Tween 20 (Sigma, St Louis, MO, USA) and 2% bovine serum albumin. The membranes were probed overnight at 4°C with the primary antibodies obtained from Cell Signaling Technology (Beverly, MA, USA): Phospho-ERK1/2, ERK1/2, phospho-IκBα and NF-KB inhibitor (IκBα). The immunoreactive bands were detected using a secondary antibody conjugated to horseradish peroxidase and a chemiluminescent detection system (ECL, Amersham, Little Chalfont, UK).

Flow cytometry (FACS) analysis

For FACS analysis, cells were grown in triplicate in six-well dishes and were incubated with *P. gingivalis* as described above. Cells were dissociated with trypsin and EDTA, washed with PBS and fixed with ice-cold 4% paraformaldehyde at 4°C. Fixed cells were then reacted with anti-gp130 mouse monoclonal immunoglobulin (Ig) G1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cells were then washed and labelled with fluoresein isothiocyanate (FITC)–goat anti-mouse IgG1. Using a FACScan (BD Biosciences, Sparks, MD, USA), fixed cells were gated by forward- and side-scatter settings that were optimized with the use of autofluorescence of untreated cells, and debris was excluded by a hardware gate on forward scatter. Normal mouse IgG1–FITC (Santa Cruz Biotechnology) was used as a negative control. A total of 10 000 events were counted for each set of conditions.

Statistical analysis

All statistical analyses were performed using one-way ANOVA with Turkey–Kramer multiple-comparisons test. Differences in the data were considered significant when the probability value was $< 5.0\%$ ($p < 0.05$).

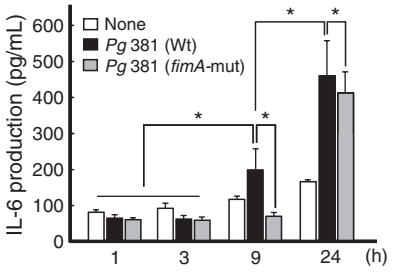

Fig. 1. Effects of IL-6 secretion by *P. gingivalis* infection in HUVEC. *Porphyromonas gingivalis* FDC 381 (wild type, wt) or *P. gingivalis* FDC 381 (*fimA* mutant) were added to HUVEC cultures at a MOI of 100 = 1, and incubated at 37°C. After *P. gingivalis* infection for the indicated times, culture supernatants were collected for IL-6 ELISA assay as described in 'Material and methods' section. Control cultures ('None') were incubated with culture media only. Data are expressed as means ± SD from three independent experiments. $*p < 0.05$ compared with control cultures (ANOVA).

Results

Increase of IL-6 secretion in *P. gingivalis*-infected HUVEC

As shown in Fig.1, we found that IL-6 secretion was not changed in *P. gingivalis* FDC381 (wt)-infected HUVEC at 1–3 h, whereas the secretion of IL-6 increased significantly after 9 h infection. The secretion of IL-6 continued to increase for 24 h after initial infection. Importantly, these increased levels were significantly higher than those for *P. gingivalis* FDC381 (*fimA* mutant)-infected HUVEC at 9 and 24 h postinfection.

Effects of ERK1/2 and NF-κB on IL-6 secretion in *P. gingivalis*-infected HUVEC

Phosphorylation of ERK1/2 was enhanced dramatically by both *P. gingivalis* (wt) and *P. gingivalis* (*fimA* mutant) infection in HUVEC (Fig. 2-A,B). Thus, to examine the effects of the ERK1/2 pathway on *P. gingivalis* FDC381 (wt)-mediated IL-6 secretion, we investigated whether the specific MEK1 inhibitor, PD98059, impairs IL-6 secretion. As shown in Fig. 2C,

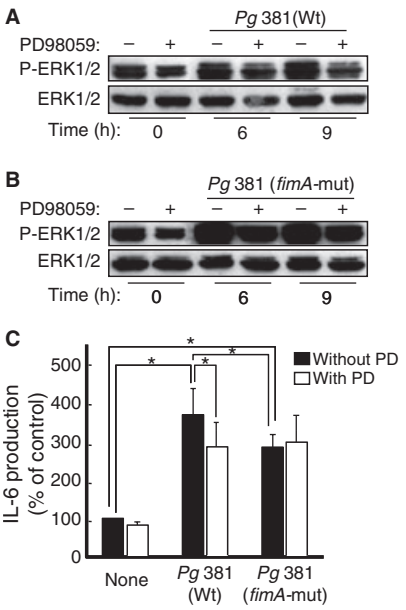

Fig. 2. Effects of ERK1/2 on IL-6 secretion stimulated by *P. gingivalis* infection in HUVEC. The HUVEC were pretreated with or without specific ERK1/2 inhibitor (10 μM PD98059) for 1 h before *P. gingivalis* infection. *Porphyromonas gingivalis* FDC 381 (wt; A) or *P. gingivalis* FDC 381 (*fimA* mutant; B), at a MOI of 100 = 1, were then added to HUVEC cultures, followed by incubation at 37°C. At the indicated times after *P. gingivalis* infection, HUVEC were lysed using cell lysis buffer. Total cell lysates were analyzed by Western blotting using antibodies to phospho-ERK1/2. Antibody to ERK1/2 was used as an internal control. Results are representative of four independent experiments. (C) Interleukin-6 ELISA assay. At an incubation time of 24 h post-*P. gingivalis* infection, culture supernatants were collected for IL-6 ELISA analysis. The control culture was incubated with culture media only. Data are expressed as means ± SD from three independent experiments. $*p < 0.05$ compared with control culture (ANOVA).

P. gingivalis (wt)-mediated IL-6 secretion was partly inhibited by pretreatment with PD98059 in HUVEC. However, there was no significant difference in IL-6 secretion with and without PD98059 during *P. gingivalis* (*fimA* mutant) infection in HUVEC.

Next, we demonstrated the time-dependent increase of phospho-IκBα with infection of wild-type *P. gingivalis* in HUVEC (Fig. 3A). Interestingly, we found, in contrast, a slight increase of

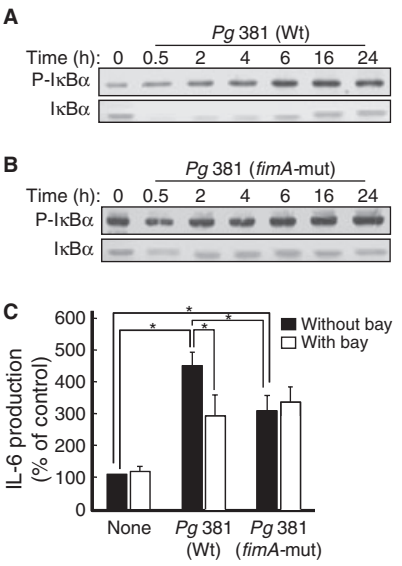

Fig. 3. Effects of NF-κB on IL-6 secretion stimulated by *P. gingivalis* infection in HUVEC. The HUVEC were pretreated with or without specific NF-κB inhibitor (2 μM Bay11-7082) for 1 h before *P. gingivalis* infection. *Porphyromonas gingivalis* FDC 381 (wt; A) or *P. gingivalis* FDC 381 (*fimA* mutant; B), at a MOI of 100 = 1, were then added to HUVEC cultures, followed by incubation at 37°C. At the indicated times after *P. gingivalis* infection, HUVEC were lysed using cell lysis buffer. Total cell lysates were analysed by Western blotting using antibodies to phospho-IκBα. Antibody to IκBα was used as an internal control. Results are representative of four independent experiments. (C) Interleukin-6 ELISA assay. At an incubation time of 24 h post-*P. gingivalis* infection, culture supernatants were collected for IL-6 ELISA analysis. The control culture was incubated with culture media only. Data are expressed as means ± SD from three independent experiments. $*p < 0.05$ compared with control culture (ANOVA).

phospho-IκBα with *fimA* mutant infection (Fig. 3B). Based on these results, in order to examine the effects of the NF-κB pathway on *P. gingivalis* (wt)-mediated IL-6 secretion, we investigated whether the NF-κB inhibitor, Bay11-7082, impairs IL-6 secretion. As shown in Fig. 3C, *P. gingivalis* (wt)-mediated IL-6 secretion was partly inhibited by pretreatment with Bay11-7082 in HUVEC. However, there was no significant difference in IL-6 secretion with and without Bay11-7082 during *P. gingivalis* (*fimA* mutant) infection in HUVEC.

Importantly, in uninfected conditions, we demonstrated that these inhibitors did not impair IL-6 secretion in uninfected HUVEC (Figs 2C and 3C).

Effects of *P. gingivalis* fimbriae on gp130 mRNA expression in HUVEC

From semi-quantitative RT-PCR analysis, we found that the expression of gp130 mRNA in *P. gingivalis* (wt)-infected HUVEC increased dramatically as early as 1–6 h after infection compared with basal levels at 0 h (Fig. 4A, left panel). Importantly, in contrast, we demonstrated that the expression of gp130 mRNA was significantly suppressed in *P. gingivalis* (*fimA* mutant)-infected HUVEC (Fig. 4A, right panel). Based on these results, we further investigated the difference of gp130 mRNA expression levels between *P. gingivalis* (wt) and *P. gingivalis* (*fimA* mutant) infection in HUVEC using qRT-PCR methods. As expected, *P. gingivalis* (wt)-infected HUVEC significantly increased the expression of gp130 mRNA as early as 1–6 h after infection, and we found that the expression of gp130 mRNA was significantly suppressed in *P. gingivalis* (*fimA* mutant)-infected HUVEC (Fig. 4B).

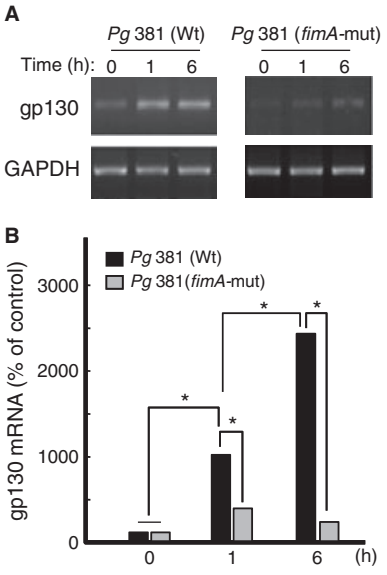

Fig. 4. Effects of *P. gingivalis* fimbriae on gp130 mRNA expression in HUVEC. Semi-quantitative RT-PCR analysis (A) and quantitative RT-PCR (qRT-PCR) analysis (B) of gp130 mRNA expression in HUVEC. *Porphyromonas gingivalis* FDC 381 (wt) or *P. gingivalis* FDC 381 (*fimA* mutant), at a MOI of 100 = 1, were added to HUVEC cultures, followed by incubation at 37°C. At the indicated times after *P. gingivalis* infection, HUVEC were collected and total RNA was extracted for semi-quantitative or quantitative RT-PCR as described in the 'Material and methods' section. Results are representative of three independent experiments. In qRT-PCR, the values of gp130 mRNA expression were analysed and expressed as relative values against those form untreated cells (0 h = 100%).

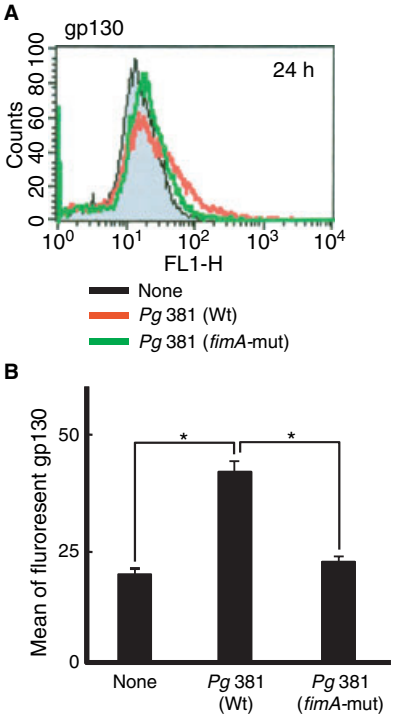

Fig. 5. Effects of *P. gingivalis* fimbriae on gp130 expression on HUVEC. *Porphyromonas gingivalis* FDC 381 (wt) or *P. gingivalis* FDC 381 (*fimA* mutant) at a MOI of 100 = 1, were added to HUVEC cultures, followed by incubation at 37°C. (A) At 24 h after *P. gingivalis* infection, HUVEC were harvested and analysed for gp130 expression by FACS as described in the 'Material and methods' section. The shadow histogram (black line) indicates the control culture; the red line indicates HUVEC infected with *P. gingivalis* FDC 381 (wt); the green line indicates HUVEC infected with *P. gingivalis* FDC 381 (*fimA* mutant). Control culture ('None') was incubated with culture media only. These results are representative of four independent experiments. (B) The mean of fluroresent of gp130 is shown. Results are expressed as means ± SD from four independent experiments. $*p < 0.05$ compared with control ('None'; paired Student's *t*-test).

Effects of *P. gingivalis* fimbriae on expression of gp130 protein on the cell surface of HUVEC

Corresponding to the results of mRNA expression shown in Fig. 4, FACS analysis revealed that the expression of gp130 increased significantly at 24 h postinfection with *P. gingivalis* (wt) in HUVEC. Interestingly, we found in contrast that the expression of gp130 was not increased by infection of *P. gingivalis* (*fimA* mutant) in HUVEC (Fig. 5A). Furthermore, by statistical analysis of fluorescence levels of gp130, we demonstrated that there was significant difference between *P. gingivalis* (wt) and *P. gingivalis* (*fimA* mutant) infection in gp130 expression in HUVEC (Fig. 5B).

Discussion

The possibility that periodontal disease might influence the morbidity of systemic diseases constitutes a research topic of great current interest (19). Several reports have suggested that persistent, low-grade periodontal infection with gram-negative bacteria is associated with increased atherosclerosis (17,18). In particular, *P. gingivalis* at a periodontal lesion has been shown to invade into aortic endothelial cells via the bloodstream, and the fimbriae are an important factor in this process (20,21). These findings indicate the critical roles of fimbriae in the virulence of *P. gingivalis*.

In the present study, we examined the effects of avirulent *P. gingivalis* fimbriae on inflammatory responses, especially cytokine regulation targeting IL-6 in endothelial cells. Interestingly, as shown in Fig. 1, we demonstrated that IL-6 secretion was dramatically enhanced in HUVEC by avirulent *P. gingivalis* (wt) infection. Furthermore, we found that the enhancement of IL-6 levels by *P. gingivalis* (wt) infection to be significantly impaired in *P. gingivalis* (*fimA* mutant)-infected HUVEC. These findings indicate that *P. gingivalis* fimbriae-

mediated signals regulate the secretion of IL-6 in HUVEC.

The ERK1/2 pathway is activated by phosphorylation on threonine and tyrosine residues by the dual specificity kinase, MEK1, which induces their translocation into the nucleus, where they activate or suppress a variety of transcription factors involved in growth and differentiation (12,22). To evaluate whether the ERK1/2 pathway is involved in the regulation of IL-6 secretion by *P. gingivalis* infection, we examined the effects of *P. gingivalis* infection on IL-6 secretion in HUVEC cultured with or without the specific MEK1 inhibitor, PD98059. As expected, we found that *P. gingivalis* (wt) infection enhanced IL-6 secretion in HUVEC (Fig. 2C). In addition, we demonstrated that PD98059 partly inhibited the enhancement of *P. gingivalis* (wt)-mediated IL-6 secretion (Fig. 2C). Next, we found that the specific NF-κB inhibitor, Bay11-7082, also partly inhibited the enhancement of *P. gingivalis* (wt)-mediated IL-6 secretion in HUVEC (Fig. 3C). Furthermore, we found a slight increase in phospho-IκBα by *fimA* mutant stimuli. In contrast, since a dramatic increase of phospho-IκBα was observed in wild-type *P. gingivalis* infection, the differences in phospho-IκBα might depend on *P. gingivalis* fimbriae. Importantly, we found that there was no significant difference of IL-6 secretion in *P. gingivalis* (*fimA* mutant)-infected HUVEC with or without PD98059 treatment (Fig. 2C). As for the inhibition assay using PD98059, we demonstrated that there was no significant difference of IL-6 secretion in *P. gingivalis* (*fimA* mutant)-infected HUVEC with or without Bay11-7082 treatment (Fig. 3C). These results suggest that the enhancement of *P. gingivalis* fimbriae-mediated IL-6 secretion is, at least in part, directly or indirectly, involved in the ERK1/2 or NF-κB signalling pathway in HUVEC.

Recently, Mao *et al.* have reported that *P. gingivalis* infection promotes the phosphorylation of janus kinases (JAK) 1 and signal transducer and activator of transcription (Stat) 3 in gingival epithelial cells (23), whereas Walter *et al.* have reported that *P. gingivalis* infection regulates two key signal transduction pathways, phosphorylation of mitogen-activated protein kinase (MAPK) p38 and nuclear translocation of NF-κB, in endothelial cells (24). In the present study, we showed that both the ERK1/2 and the NF-κB pathway are important in regulating the *P. gingivalis* fimbriae-mediated IL-6 autocrine mechanism in HUVEC. However, other signalling molecules might be activated in *P. gingivalis*-infected HUVEC, because PD98059 or Bay11-7082 partly inhibits the IL-6 secretion in *P. gingivalis* (wt)-infected HUVEC. To clarify the effects of other *P. gingivalis* fimbriae-dependent signals on the IL-6 autocrine mechanisms in endothelial cells, further experiments will be needed.

Pro-inflammatory cytokines, such as IL-6, have been implicated in the pathogenesis of atherosclerosis (25,26). In addition, in a murine model of atherosclerosis, it has been reported that atherosclerotic lesion size correlates with IL-6 levels (27). Furthermore, circulating levels of IL-6 have been shown to correlate with an adverse prognosis for patients suffering from coronary artery disease (14). Although the detailed mechanisms of how cytokines may accelerate the development of atherosclerosis remain poorly understood, finding these mechanisms could be essential to establishment of anti-atherosclerotic treatment strategies depending on circulating levels of IL-6.

It has been widely accepted that gp130 is a common receptor for the IL-6 family, such as IL-11, leukaemia inhibitory factor (11,12). Considering the autocrine mechanism of IL-6 in development of atherosclerotic lesions by endothelial cells, we examined whether expression of gp130 could be enhanced by *P. gingivalis* infection in HUVEC. At first, as shown in Fig. 4A,B, we demonstrated, for the first time, that *P. gingivalis* (wt) infection significantly enhances gp130 mRNA expression in HUVEC. Corresponding to the mRNA expression, gp130 protein expression on the cell surface was also enhanced 24 h after *P. gingivalis* (wt) infection (Fig. 5A,B). Next, we found that there were no significant differences in gp130 expression between no infection and *P. gingivalis* (*fimA* mutant) infection (Fig. 5). These results might indicate

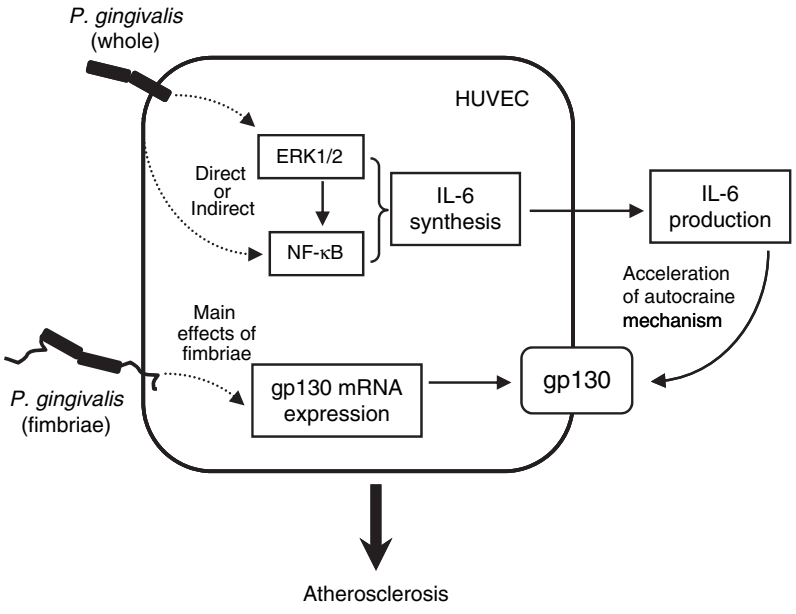

Fig. 6. Schematic representation of enhancement of *P. gingivalis*-mediated IL-6 or gp130 expression in HUVEC. Interleukin-6 production is, at least in part, dependent on the *P gingivalis* infection in HUVEC via the ERK1/2 or the NF-κB pathway. *Porphyromonas gingivalis* fimbriae are, for the most part, involved in gp130 expression. These findings might explain the autocrine mechanisms of IL-6 in HUVEC infected by *P. gingivalis*.

that *P. gingivalis* FDC381 fimbriae enhance the gp130 expression in HUVEC. Recently, Luchtefeld *et al.* reported that the gp130-driven acute phase reaction is a critical regulator of the process of atherosclerotic plaque development in both mice and humans (28). This report might support the finding that enhancement of gp130 expression is involved in development of atherosclerosis indirectly.

Several reports have noted that inflammation of the endothelium is one of the key factors in the progression of atherosclerosis (7,20,21). Our findings have shown the possible mechanism that fimbriae-dependent *P. gingivalis* invasion into HUVEC regulates the IL-6 autocrine system (Fig. 6). Periodontal disease is known to influence the systemic condition in various ways, and the bacteria and their products, such as fimbriae, may spread from periodontal lesions via the systemic circulation to affect distant organs. The pathway shown in the present study could be an attractive target for atherosclerotic lesions in patients with chronic periodontitis, and contribute to development of the concept of periodontal medicine.

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