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Peptidylarginine deiminase from *Porphyromonas gingivalis* (PPAD) contributes to infection of gingival fibroblasts and induction of PGE2-signaling pathway

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SUMMARY

Porphyromonas gingivalis (*Pg*) expresses the enzyme peptidylarginine deiminase (PPAD), which has a strong preference for C-terminal arginines. Due to the combined activity of PPAD and Arg-specific gingipains, *Pg* on the cell surface is highly citrullinated. To investigate the contribution of PPAD to the interaction of *Pg* with primary human gingival fibroblasts (PHGF) and *Pg*-induced synthesis of prostaglandin E2 (PGE2), PHGF were infected with wild-type *Pg* ATCC 33277, an isogenic PPAD-knockout strain (Δ*ppad*) or a mutated strain (C351A) expressing an inactive enzyme in which the catalytic cysteine has been mutated to alanine ($PPAD^{C351A}$). Cells were infected in medium containing the mutants alone or in medium supplemented with purified, active PPAD. PHGF infection was assessed by colony-forming assay, microscopic analysis and flow cytometry. Expression of COX-2 and mPGES-1, key factors in the prostaglandin synthesis pathway, was examined by qRT-PCR, while PGE2 synthesis was evaluated by EIA. PHGF were infected more efficiently by wt-*Pg* than the Δ*ppad* strain, which correlated with strong induction of COX-2 and mPGES-1 expression by wt-*Pg*, but not by the PPAD activity-null mutant strains (ΔPPAD and C351A). The impaired ability of the ΔPPAD strain to adhere to and/or invade PHGF and both ΔPPAD and C351A to stimulate the PGE2-synthesis pathway was fully restored by the addition of purified PPAD. The latter effect was strongly inhibited by aspirin. Collectively, our results implicate PPAD activity, but not PPAD itself, as an important factor for gingival fibroblast infection and activation of PGE2 synthesis, the latter of which may strongly contribute to bone resorption and eventual tooth loss.

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Conflicts of interest

None declared.

Keywords

P. gingivalis; peptidylarginine deiminase; citrullination; prostaglandin E2; gingival fibroblasts

INTRODUCTION

Periodontal disease (PD) is a major public health problem affecting over 30% of the adult population worldwide. The disease is clinically characterized by chronic inflammation of tooth-supporting structures, destruction of the periodontal ligament, the resorption of alveolar bone and formation of periodontal pockets. Untreated periodontitis may lead to tooth loss (Eke *et al.*, 2012). One pathogen recognized as a principal causative agent of PD is the anaerobic, non-motile, asaccharolytic, Gram-negative bacterium, *Porphyromonas gingivalis* (*Pg*). The presence of *Pg* in subgingival plaque correlates with disease severity, as assessed by attachment loss, periodontal pocket depth and bleeding on probing (Bostanci & Belibasakis, 2012).

A wide variety of virulence factors, including lipopolysaccharides (LPS), fimbriae, proteinases (gingipains), haemagglutinins and haemolysins contribute to the pathogenicity of *Pg* (Bostanci & Belibasakis, 2012). Recently, considerable interest has been focused on peptidylarginine deiminase expressed by *Pg* (PPAD) (Wegner *et al.*, 2010; Maresz *et al.*, 2013). PPAD is able to modify proteins by deimination of peptidylarginine residues to produce peptidylcitrulline and ammonia (McGraw *et al.*, 1999; Rodriguez *et al.*, 2009). Unlike mammalian PADs, which act only on Arg within the polypeptide chain in a calcium-dependent manner, PPAD primarily citrullinates C-terminal residues and can also deiminate free L-arginine in the absence of calcium (Rodriguez *et al.*, 2009; Bicker & Thompson, 2013).

The conversion of positively charged arginine into neutral citrulline may affect the folding and stability of proteins and peptides, alter their susceptibility to proteolysis and abrogate their biological activity. For example, citrullination of CXCL8 results in a considerable reduction in binding to glycosaminoglycans and prevents proteolytic truncation of the chemokine by plasmin or thrombin, thus precluding the ability of CXCL8 to recruit neutrophils (Loos *et al.*, 2008; Proost *et al.*, 2008; Loos *et al.*, 2009). In turn, citrullination of the antibacterial peptide LL-37 by human PAD2 and PAD4 compromises its ability to neutralize lipopolysaccharides and makes the peptide more prone to degradation by proteases. Additionally, citrullination alters the immunomodulatory functions of LL-37 that are essential for the prevention of endotoxin-induced sepsis (Kilsgard *et al.*, 2012, Koziel *et al.*, 2014) and may upset other regulatory activities of this and other host defense peptides (Choi *et al.*, 2012; Nijnik *et al.*, 2012; Pulido *et al.*, 2012; Semple & Dorin, 2012; Sall *et al.*, 2013). Several recent reports indicate that protein citrullination catalyzed by PPAD may contribute to the pathogenesis of periodontitis and rheumatoid arthritis (RA). To the latter, citrullination of bacterial and host proteins by PPAD in inflamed gingival tissues is considered a molecular mechanism for generating antigens that initiate and/or enhance the autoimmune response in rheumatoid arthritis (Wegner *et al.*, 2010; Nesse *et al.*, 2012; Maresz *et al.*, 2013). Moreover, it was recently reported that PPAD efficiently citrullinates

the C-terminal arginine of epidermal growth factor (EGF), which subsequently impairs its biological activity (Pyrc *et al.*, 2012). Decreased activity of EGF in gingival pockets may at least partially contribute to the tissue damage and delayed healing of the periodontium observed during *Pg* infection.

Chronic periodontitis, which is now recognized as a pathogen-driven dysbiotic disease (Hajishengallis & Lamont, 2012; Wright *et al.*, 2013), entails multiple cycles of progression and remission mediated by the modulation of pro-inflammatory signaling networks (Demmer & Papapanou, 2010). One inflammatory mediator at the interface between bacterial infection and periodontal tissue damage is prostaglandin E2 (PGE2) (Offenbacher *et al.*, 1986, 1993; Noguchi & Ishikawa, 2007; Taxman *et al.*, 2012). PGE2 has several biological functions, including vasodilation and enhanced vascular permeability; however, in the context of the pathology of periodontitis, the induction of osteoclastogenesis is its most important function (Lerner, 1991, Brechter & Lerner, 2007). To this end, the correlation between PGE2 levels in the gingival crevicular fluid (GCF) and clinical parameters of periodontitis, such as periodontal attachment loss and bleeding on probing, is clearly documented (Preshaw & Heasman, 2002; Noguchi & Ishikawa, 2007; Zhong *et al.*, 2007; Zhang *et al.*, 2011; Taxman *et al.*, 2012). However, the impact of PPAD on PGE2 signaling has not yet been elucidated.

The present study was undertaken to evaluate whether PPAD modulates prostaglandin signaling. To this end, we showed that PPAD activity, but not the protein alone, contributed to the infection of PHGF by *Pg* and activation of the PGE2 synthesis pathway, which is manifested by increased levels of COX-2 and mPGES-1 expression as well as significantly enhanced secretion of PGE2. The effect was linked at least partially to the citrullination of bacterial/host cell surface proteins and may contribute to alveolar bone loss at infected periodontitis sites.

MATERIALS AND METHODS

Construction of the *P. gingivalis* ΔPPAD mutant (ATCC 33277)

The mutant of *P. gingivalis* ATCC harboring a *ppad* gene deletion (Genbank accession number 188594442; locus tag PGN_0898) was obtained as described previously for the W83 strain (Wegner *et al.*, 2010). Erythromycin-resistant clones were subcultured on selective plates and genomic integration was confirmed by PCR using primers flanking the integration site.

Plasmid construction for PPAD C351A (ATCC 33277) active site-directed inactivation

Three DNA fragments were amplified by PCR: 1.3 kb from the 5′ end of the *ppad* gene (TIGR accession no. PG1424; primers used were pUPPADa_F and pUPPADa_R), the tetracycline cassette (tetQ; primers used were tetQ_inf F and tetQ_PPAD_inf R), and a 960 bp fragment flanking the 3′ end of the *ppad* gene (primers used were pUPPADb_F and pUPPADb_R). The DNA sequences were cloned into the pUC19 plasmid using InFusion Cloning HD Kit (Clontech) in the *E. coli* DH5α strain. In the obtained plasmid, the PPAD-encoding sequence was further modified by substitution of the catalytic residue Cys351 with

alanine to create the final plasmid PPAD_C351A. This was accomplished using the SLIM method (primers used were C351AFs, C351ARs, C351AFl and C351ARl) (Chiu *et al.*, 2004). The correctness of the obtained constructs was confirmed by sequencing. The PPAD C351A plasmid was used for electroporation of *P. gingivalis* ATCC 33277 (Smith *et al.*, 1990). The correct placement and orientation of the DNA segments were confirmed by sequencing and PPAD activity assay (Boyde & Rahmatullah, 1980).

Expression and purification of *P. gingivalis* PPAD

The *Pg* W83 strain was engineered to secrete PPAD in a soluble form using the same molecular strategy reported previously for the RgpB protease (Zhou *et al.*, 2013). Subsequently, PPAD was purified from the culture medium via ion-exchange and gel filtration chromatography, as described previously (J. Potempa – unpublished data). The purity of PPAD was evaluated by SDS-PAGE followed by silver staining. PPAD activity was tested using a colorimetric assay, as described previously (Liao *et al.*, 2005) with N_{α}-benzyloarginine ethyl ester (BAEE) as a substrate. Activity was expressed as mU/μL (1 mU = 1 nmol of citrulline produced within 1 h of the reaction).

Bacterial culture

P. gingivalis strains (wt-*Pg* ATCC 33277 and its isogenic mutant Δ*ppad* and C351A expressing catalytically inactive PPAD ($PPAD^{C351A}$) were grown on blood agar plates (BHI, brain heart infusion medium supplemented with 5% sheep blood, 5 μg mL^{-1} hemin and 0.5 μg mL^{-1} vitamin K in an anaerobic chamber (90% N_2, 10% CO_2, and 5% H_2). The media were additionally supplemented with erythromycin (5 μg mL^{-1}) or tetracycline (1 μg mL^{-1}) for the growth of ΔPPAD or C351A mutants, respectively. After cultivation at 37°C for 7 days, bacterial cells were inoculated into enriched BHI broth (Becton Dickinson) for overnight culture. Prior to inoculation, bacteria were washed with phosphate-buffered saline (PBS) and resuspended in a fresh culture medium. Bacterial cell counts were determined using a spectrophotometer; an optical density of 1.0 at 600 nm corresponded to a concentration of 1×10^9 colony-forming units (CFU) per mL. Bacterial suspensions were used for further experiments.

Gingival fibroblast isolation and culture

Gingival biopsies were collected from healthy subjects presenting for orthodontic treatment at the Department of Periodontology and Oral Medicine, Jagiellonian University, Medical College, Institute of Dentistry in Krakow, Poland. The study was approved by the Bioethical Committee of the Jagiellonian University in Krakow, Poland (KBET/310/B/2012). Prior to the study written informed consent was obtained from all donors. Briefly, following enzymatic separation of the epithelial layer, the remaining connective tissue was digested with 0.1% collagenase I (Invitrogen). After digestion, the cell pellet was plated in a T25 flask in Dulbecco's Modified Eagle Medium (DMEM, PAA GmbH) supplemented with 10% heat-inactivated foetal bovine serum (FBS, PAA), penicillin/streptomycin (50 U mL^{-1}), gentamicin (50 U mL^{-1}), and nystatin (10 μg mL^{-1}, PAA), at 37°C in a humidified atmosphere containing 5% CO_2. Immunofluorescent staining with anti-vimentin and anti-cytokeratin antibodies was conducted on samples from cultures at the second passage to

evaluate the homogeneity of fibroblast cultures. Cell viability was confirmed by Trypan blue staining to be 95–98%. Tests for *Mycoplasma spp*. were negative. Fibroblast cultures at passages 3–6 were used for experiments.

Colony-forming assay

Gingival fibroblasts were grown until confluence in a T75 flask in DMEM supplemented with 10% FBS, penicillin/streptomycin (50 U mL^{-1}), and gentamicin (50 U mL^{-1}) at 37°C in a humidified atmosphere containing 5% CO_2. Twenty hours before the experiment, cells were trypsinized and plated in a fresh culture media (DMEM, 2% FBS) in a 24-well plate at 9.5×10^5 cells per well. The next day, cells were infected with bacterial suspensions prepared from overnight cultures. In brief, overnight bacterial cultures were centrifuged, rinsed with PBS and adjusted to the appropriate MOI in fresh culture media. Cells were infected with wt-*Pg* ATCC 33277 and its isogenic mutant Δ*ppad* at an MOI of 100 for 1.5 h, 3 h, and 6 h, respectively.. To assess adhesion and invasion, infected cells were rinsed three times with PBS and lysed. Cell lysates were serially diluted and seeded on blood agar plates. After 10 days of growth in an anaerobic atmosphere at 37°C, bacterial colonies were counted and the results were expressed as CFU per mL. To evaluate bacterial invasion into fibroblasts, an antibiotic protection assay was performed. Immediately after infection, the cells were rinsed with PBS followed by 1.5 h incubation with gentamicin (100 μg mL^{-1}). Subsequent steps were performed as described above (adhesion and invasion assay).

Supplementation of PPAD mutants with purified, active enzyme

Gingival fibroblasts were infected for 3 h at an MOI of 100 with the PPAD mutants (ΔPPAD and C351A) in medium supplemented with purified PPAD at 0.066 mU μL^{-1} (for a total of 66 mU), which corresponds to the mean PPAD activity of 20 h cultures of wt-*Pg* adjusted to *Pg* cell numbers used at an MOI of 100.

FACS and microscopic analysis of *P. gingivalis* adhesion to and invasion of PHGF

Overnight cultures of wt-*Pg* and the Δ*ppad* strain (ATCC 33277) were centrifuged, rinsed in PBS and resuspended in PBS to an OD of 1.5 in a total volume of 2 × 10 mL each. Next, bacterial cultures were labeled with fluorescent dye (Cell Trace CFSE Cell Proliferation Kit for Flow Cytometry, Life Technologies). In brief, CFSE dye dissolved in DMSO was diluted in PBS to a final concentration of 10 μM, mixed with bacterial pellets and incubated for 15 min at 37°C (in the dark). After incubation, bacterial pellets were rinsed several times with fresh PBS. Bacterial cell numbers were adjusted to 1×10^9 CFU per mL (OD = 1.0 at 600 nm). Primary human gingival fibroblasts (PHGF) were plated in fresh culture medium (DMEM, 2% FBS) at a density of 4×10^5 cells per well in a 24-well plate. The next day, cells were infected for 3 h at an MOI of 100 with bacterial suspensions prepared, as described earlier. Infection was conducted in three different ways. In the first set of experiments, infected cells were trypsinized, resuspended in fresh RPMI-1640 (Euroclone) supplemented with 0.5% FBS and analyzed using a FACScan flow cytometer (Becton Dickinson). Analysis was performed using CellQuest software to determine the percentage and mean fluorescence intensity of positive, infected cells. Two remaining sets of experiments were performed to assess adhesion and invasion or invasion alone using a

fluorescent microscope. Bacterial invasion was assessed by employing an antibiotic protection assay, as described above. Infected cells were fixed in 4% formaldehyde, rinsed with PBS and analyzed with a fluorescent microscope (Eclipse E600, Nikon Inc.). Photographic documentation was obtained with a digital camera controlled by NIS-Elements software (Nikon Inc.). Analysis of adhesion and invasion or invasion only by fluorescently labeled bacteria was performed by quantification of the mean intensity of fluorescence within a defined area of interest (AOI) and expressed as arbitrary units [*I/pix*]. Evaluation of 12 AOIs was performed per experimental condition. The analysis was conducted with Imaging Software NIS Elements BR3.2.

Evaluation of PGE2 signaling

Gingival fibroblasts were cultured in DMEM, 10% FBS, penicillin/streptomycin and gentamicin until confluence. The day before the experiment, cells were trypsinized and plated in fresh DMEM supplemented with 2% FBS in a 6-well plate at a density of 9.5×10^5 cells per well. On the following day, cells were infected at an MOI of 100 for 3 h with wt-*Pg*, the Δ*ppad* and C351A mutant strains, and PPAD mutants were supplemented with purified PPAD. In parallel experiments, cells were infected for 3 h in the same way but in the presence of aspirin (50 μg mL^{-1}), an inhibitor of cyclooxygenases. At specific times after infection, culture medium was collected, centrifuged and used for further analysis. Cells were rinsed three times with PBS and lysed using TriReagent solution. Collected lysates were used for the purification of total RNA and analysis of COX-2 and mPGES-1 gene expression by quantitative-RT PCR (qRT-PCR), as described below.

PGE2 synthesis

PGE2 synthesis was evaluated in culture media using a commercially available EIA kit (Cayman Chemical) following the manufacturer's instructions.

Gene expression analysis

Total cellular RNA was extracted using TriReagent solution (Life Technologies), according to the manufacturer's protocol. Isolated RNA was reverse transcribed with a high-capacity cDNA reverse transcription kit (Life Technologies) according to the manufacturer's protocol, using 400 ng of previously extracted RNA in a total volume of 20 μL. Quantitative real-time PCR amplification was conducted with 2 × SYBR green mix (Sigma Aldrich, Poland). The reaction was carried out in a total volume of 10 μL in the presence of forward and reverse primers and template DNA (2 μL). The following primers were used: for the amplification of β-actin, β-act5 (5′-CCACACTGTGCCCATCTACG-3′), and β-act3 (5′-AGGATCTTCATGAGGTAGTCAGTCAG-3′); for the amplification of COX-2, 5_COX-2 (5′-AGCCCTTCCTCCTGTGCCT-3′) and 3_COX-2 (5′-TCCATTTTTCGTCGAAGGACTAA-3′); for the amplification of mPGES-1, 5_mPGES-1 (5′-CACGCTGCTGGTCATCAAGAT-3′) and 3_mPGES-1 (5′-TCCTACGGGACTCTGTGCC-3′). Each primer was used at 500 nM. ROX was used as a reference dye. The PCR cycling conditions were an initial denaturation for 5 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 55°C and 45 s at 72°C. Real-time amplification was followed by melting-curve assessment to confirm product identity.

RESULTS

PPAD is involved in efficient interaction with, adhesion to and invasion of gingival fibroblasts by *P. gingivalis*

The absence of *Pg* surface protein citrullination in the Δ*ppad* strain and/or inability to citrullinate host cell surface proteins may affect its interaction with cells. Therefore, we first compared the efficiency of infection of gingival fibroblasts by wt-*Pg* and the ΔPPAD mutant strain. In all tested conditions at an MOI of 100, wt-*Pg* adhered to and invaded gingival fibroblasts more efficiently than the Δ*ppad* strain (Table 1). Of note, regardless of the level of PPAD activity, infection at an MOI of 500 for 3 h resulted in progressive cell death, i.e., apoptosis of fibroblasts (data not shown).

The ability of wt-*Pg* and the Δ*ppad* strain to infect fibroblasts was further investigated using fluorescently labeled bacteria (Fig. 1). Fluorescent microscopy (Fig. 1A and B) and flow cytometry (Fig. 1C and D) analysis revealed that the ΔPPAD mutant adhered to and invaded gingival cells to a considerably lower degree than the parental strain. This impaired ability of the ΔPPAD mutant to infect cells was independent of fibroblast donor (Fig. 1D). These data indicate that PPAD is involved in the interaction of *P. gingivalis* with gingival fibroblasts and contributes to the effective adhesion to and invasion of gingival fibroblasts by this periodontopathogen.

External addition of active PPAD restored the ability of ΔPPAD to adhere to/invade fibroblasts

To verify the role of PPAD in the adhesion to and invasion of gingival fibroblasts, purified enzyme in an amount equivalent to that of the infection dose of wt-*Pg* was added to the medium together with the ΔPPAD mutant. As shown in Figure 2, the addition of exogenous PPAD restored the ability of the *Pg* mutant to adhere to and invade fibroblasts. This result excluded a possible polar effect of *ppad* gene deletion in the mutant strain phenotype and fully confirmed the role of PPAD in the interaction of *Pg* with fibroblasts.

PGE2 signaling is affected by PPAD upon infection of gingival fibroblasts with *P. gingivalis*

Next, we attempted to verify whether PPAD can affect an immune response through the modulation of the prostaglandin-dependent pathway. As depicted in Figure 3, the infection of fibroblasts with the wt-*Pg* strain upregulated the expression of two key enzymes that participate in PGE2 synthesis, cyclooxygenase-2 (COX-2; Fig. 3A) and microsomal PGE synthase-1 (mPGES-1; Fig. 3B), and increased the synthesis of PGE2 (Fig. 3C). Remarkably, upregulation of both enzymes was strongly reduced in cells infected with the Δ*ppad* strain and the strain C351A, which expresses a catalytically inactive form of the enzyme (Fig. 3A and B). In agreement with this, the level of synthesized PGE2 was significantly lower in the conditioned media of cells infected with the mutant strains compared to the media of cells infected with wt-*Pg* (Fig. 3C). Significantly, the addition of purified PPAD into the culture media during incubation of the mutant strain with fibroblasts restored the expression of COX-2 and mPGES-1 as well as the synthesis of PGE2 to levels comparable to those observed during infection with wt-*Pg*. These results clearly argue that

PPAD activity is important for the stimulation of the prostaglandin synthesis pathway in *Pg*-infected fibroblasts.

Activation of the prostaglandin synthesis pathway in infected fibroblasts is inhibited by aspirin

Aspirin is an effective anti-inflammatory drug which affects prostaglandin pathway through non-selective, irreversible inhibition of cyclooxygenases (Fuster & Sweeny, 2011; Funosas *et al.*, 2012; Schmidt *et al.*, 2014; Shiloah *et al.*, 2014; Therefore, to validate PPAD activity-dependent stimulation of the prostaglandin synthesis pathway by *P. gingivalis*, fibroblasts were infected with *Pg* strains in combination with externally added PPAD and treated with aspirin. In all cases, aspirin reduced the expression of COX-2 (Fig. 4A) and mPGES-1 (Fig. 4B) and inhibited PGE2 synthesis (Fig. 4C). Together, these results fully confirm upregulation of the PGE2 synthesis pathway in infected fibroblasts and the importance of PPAD activity in this process.

DISCUSSION

In the oral cavity, the gingival epithelium acts as a barrier that prevents the intrusion of oral bacteria into subepithelial tissues (Amano, 2007). A number of reports show that *P. gingivalis*, a primary aetiological agent of PD, is able to enter gingival epithelial cells. More importantly, it has been shown that this periodontopathogen spreads through the upper layers of the gingival epithelial barrier, penetrates the basement membrane and invades deeper into the connective tissue, where fibroblasts are the most prevalent cell type (Yilmaz *et al.*, 2006; Amano, 2007). To date, the impact of PPAD on bacterial adhesion to and invasion of gingival cells, particularly gingival fibroblasts, has not been elucidated.

In this report, we show unambiguously that PPAD significantly contributes to the interaction of *Pg* with gingival fibroblasts. In comparison to the parental ATCC 33277 strain, the isogenic Δ*ppad* strain showed a two-fold decrease in the ability to adhere to and/or invade fibroblasts. Significantly, the addition of purified, active PPAD to the culture medium in an amount equivalent to that carried by wt-*Pg* reestablished the adhesive/invasive phenotype of the mutant, eliminating the possibility that the attenuated adhesion/invasion phenotype is due to a polar effect of *ppad* gene deletion. This argues for the genuine involvement of PPAD activity in *Pg* invasion of fibroblasts.

To date, *Pg* major fimbriae are believed to mediate the bacterial invasion of several human epithelial cell lines and contribute to the persistence of *Pg* at intracellular locations *in vitro* (Yilmaz *et al.*, 2006; Nagano *et al.*, 2013). Moreover, different types of fimbriae possess various potential levels of virulence. *Pg* fimbriae are classified into six types (types I to V and Ib) based on the genes encoding FimA, a major subunit of fimbriae. Nakagawa *et al.* (2006) reported that amongst six representative strains possessing the different types of fimbriae, strain OMZ314, which has type II fimbriae, adhered to and invaded epithelial cells to a significantly greater degree than the other strains. In this study, we used the strain ATCC 33277, which expresses less virulent type I fimbriae, but nevertheless, actively participating in the adhesion to and invasion of host cells (Lamont *et al.*, 1995; Nakagawa *et al.*, 2006; Wang *et al.*, 2007). In this context, our finding that PPAD knockout strongly

attenuated the capacity of *Pg* to adhere to and invade fibroblasts, regardless of unaffected fibrination (data not shown), is very interesting and leads us to speculate that citrullination of fimbriae significantly contributes to the fimbriae-mediated effects of *Pg* interaction with fibroblasts. Experiments to verify this assumption are in progress in our laboratories.

It has been shown that both dead and viable *Pg* (Steffen *et al.*, 2000), as well as *Pg*-derived LPS (Noguchi *et al.*, 1996), elicited the production of PGE2 in human gingival fibroblast cultures. We confirmed this finding by showing that *P. gingivalis* strongly stimulated the expression of COX-2 and mPGES-1, two key enzymes in the prostaglandin synthesis pathway, enhanced the synthesis of PGE2 in infected fibroblasts. Interestingly, this stimulation was dependent on PPAD activity, since the *Pg* strain (C351A) expressing a catalytically inactive form of the enzyme was as ineffective for activation of the prostaglandin synthesis pathway as the PPAD-null mutant (Fig. 3). The difference was highly significant for each tested component of the prostaglandin pathway but most profound in the difference between COX-2 stimulation by wt-*Pg* and the PPAD activity-deficient mutants. Significantly, supplementation of the infection medium with purified active enzyme restored the expression of COX-2 and mPGES-1 as well as production of PGE2 in mutant-infected fibroblasts to the levels induced by wt-*Pg*. Because the addition of active PPAD alone to sham-infected control fibroblast cultures did not induce the cells to produce PGE2, it appears that citrullinated *Pg* cell surface protein(s) act as ligand(s) for activating the prostaglandin synthesis pathway.

Citrullination-dependent activation of fibroblasts, the predominant cell type in periodontal connective tissue, produces excessive amounts of prostaglandins, in particular PGE2, which may have dire consequences on homeostasis in the periodontium. PGE2 plays a significant role in the inflammatory response by contributing to the pathogenesis of several chronic inflammatory conditions (Offenbacher *et al.*, 1993; Yucel-Lindberg *et al.*, 1999; Preshaw & Heasman, 2002). Specifically, PGE2 is implicated in the pathogenesis of PD. Increased levels of PGE2 are present in periodontal tissue and the GCF of patients suffering from periodontitis (Offenbacher *et al.*, 1986; Offenbacher *et al.*, 1993; Preshaw & Heasman, 2002) and strongly correlate with disease severity as measured by attachment loss. Furthermore, the involvement of PGE2 in periodontitis is supported by findings that treatment with non-steroidal anti-inflammatory drugs (NSAIDs), as well as selective COX-2 inhibitors known to inhibit PGE2 synthesis, decreased PD severity as measured by alveolar bone resorption (Offenbacher *et al.*, 1986; Yucel-Lindberg *et al.*, 1999; Lerner & Lundberg, 2002). Aspirin, a widely used NSAID, is an irreversible inhibitor of cyclooxygenases (Fuster & Sweeny, 2011). It was shown that aspirin stimulated an increase in attachment in a select group of adult smokers during long-term aspirin intake and non-surgical periodontal therapy (scaling and root planning) (Shiloah *et al.*, 2014). In another study, aspirin was the most effective NSAID for reducing probing depth, gingival index and bleeding on probing (Funosas *et al.*, 2012). In this context, our finding that aspirin obviates *Pg*-stimulated activation of the prostaglandin synthesis pathway in oral fibroblasts is of great importance since it provides insight into the mechanism of its beneficial effects on periodontal health.

In conclusion, we demonstrate here for the first time that PPAD activity contributes to *Pg* adherence to and invasion of gingival fibroblasts. Furthermore, we found that PPAD activity

through the citrullination of presently unidentified *Pg* surface protein(s) activates the prostaglandin synthesis pathway and secretion of PGE2 in infected fibroblasts. Taking into account the proven role of PGE2 in bone resorption, PPAD emerges from this study as an important virulence factor and valid target for drug development.

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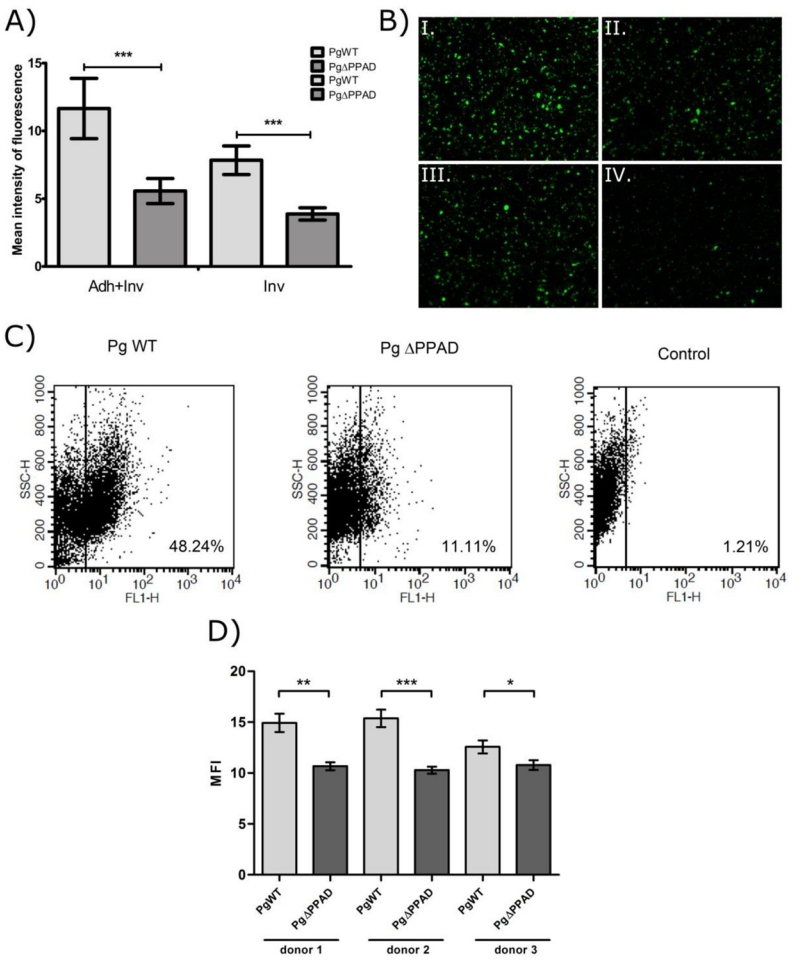

Figure 1. *P. gingivalis* adheres to and invades primary human gingival fibroblasts (PHGF) more efficiently than *P. gingivalis*-ΔPPAD mutants

Human fibroblasts were infected with CFSE-labeled wt-*Pg* or the ΔPPAD mutant. (A) Mean intensity of fluorescence for adhesion/invasion (Adh+Inv) or invasion only (Inv) within a defined area of interest (AOI) was determined and expressed as arbitrary units [*I/pix*]. (B) Representative images from microscopic analysis of adhesion/invasion (I. and II.) and invasion only (III. and IV.) by wt-*Pg* (I. and III.) and the Δ*ppad* strain (II. and IV.) are shown. Magnification = 200×. (C) Dot plots of the percentage of infected cells and (D) the mean fluorescence intensity (MFI) of infection (adhesion and invasion) determined by flow cytometry analysis of human fibroblasts infected with CFSE-labeled wt-*Pg* and Δ*ppad* strains. In all experiments, PHGF were infected for 3 h at an MOI of 100, and all experiments were performed three times. Results are expressed as means ± SD (***, $p < 0.001$; **, $p < 0.01$; and *, $p < 0.05$).

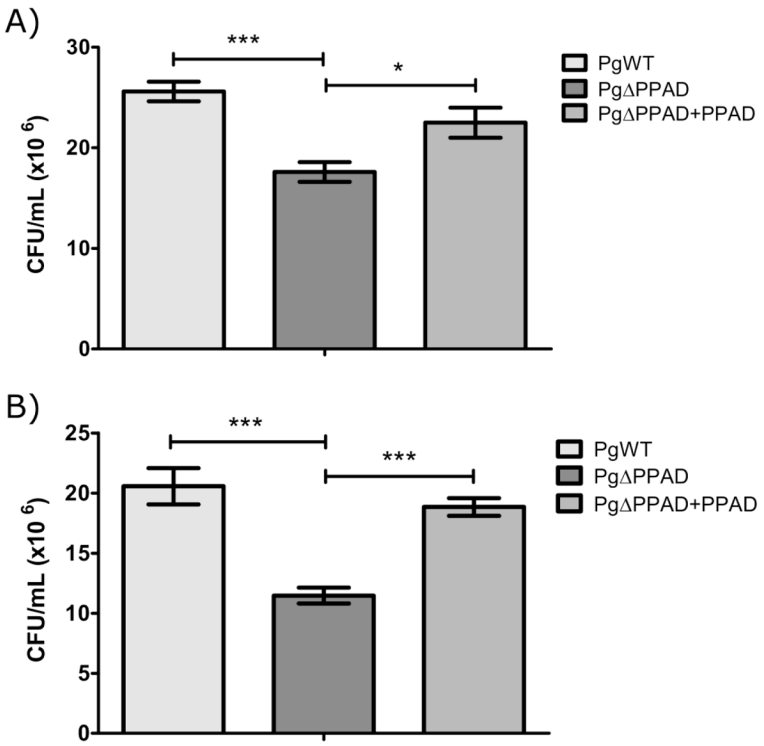

Figure 2. PPAD supplementation restores the ability of *P. gingivalis* ΔPPAD to adhere to and invade primary human gingival fibroblasts (PHGF)

Purified PPAD (0.066 mU/μL, total 66 mU) was added to a culture medium of PHGF cells during infection with the ΔPPAD mutant, and adherence/invasion (A) and invasion alone (B) were determined. In all experiments, PHGF were infected for 3 h at an MOI of 100. Data represent three independent experiments and are expressed as means ± SD (***, $p < 0.001$; **, $p < 0.01$; and *, $p < 0.05$).

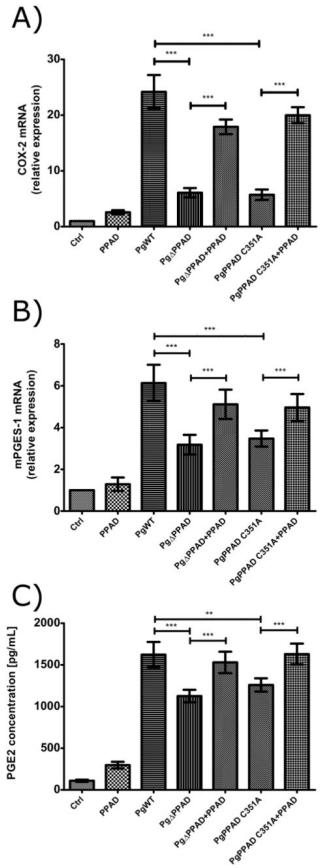

Figure 3. PPAD induces prostaglandin E2 (PGE2) signaling in primary human gingival fibroblasts (PHGF)

Relative expression levels of cyclooxygenase-2 (COX-2) (A) and microsomal PGE synthase-1 (mPGES-1) (B), and the concentration of PGE2 (C) in PHGF infected with wt-*P*g, ΔPPAD and C351A mutant strains for 3 h at an MOI of 100. Infection with mutant strains was also performed in the presence of purified PPAD (0.066 mU/μL, total 66 mU). Quantitative real-time PCR was performed using β-actin as a reference gene. PGE2 concentrations were evaluated by EIA test. Data represent three independent experiments and are expressed as means ± SD (***, $p < 0.001$; **, $p < 0.01$; and *, $p < 0.05$).

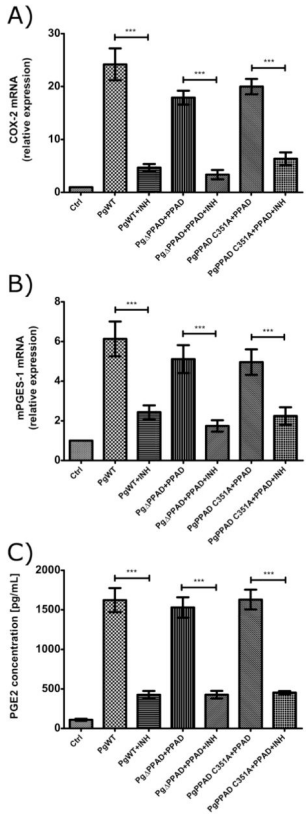

Figure 4. PPAD-induced prostaglandin E2 (PGE2) synthesis in primary human gingival fibroblasts (PHGF) is inhibited by aspirin

PHGF infection for 3 h at an MOI of 100 with wt-*P*g or PPAD mutants (ΔPPAD and C351A) supplemented with purified PPAD (0.066 mU/μL, total 66 mU) resulted in an increased expression of cyclooxygenase-2 (COX-2) (A), microsomal PGE synthase-1 (mPGES-1) (B) and upregulated synthesis of PGE2 (C). The addition of aspirin (INH, 50 μg mL^{-1}) to infected cells caused inhibition of PPAD-induced PGE2 signaling. Experiments were performed three times and data are expressed as means ± SD (***, $p < 0.001$; **, $p < 0.01$; and *, $p < 0.05$).

Table 1

Comparison of adhesion/invasion or invasion alone of primary human gingival fibroblasts (PHGF) by *P. gingivalis* (ATCC 33277)

Cells were infected with 10^8 CFU (MOI = 100) of wt-*Pg* and the Δ*ppad* strain for 1.5 h, 3 h and 6 h. Infected cell monolayers were washed and cells were lysed. Lysates were serially diluted and plated on blood agar, and CFU were counted after 10 days of cultivation. Invasion was assessed using an antibiotic protection assay. Results are expressed as means ± SD and represent three independent experiments.

Strain	Inoculum [CFU]	Infection time	Adhering and invading bacteria		Invading bacteria only	
			[CFU]	Student *t* test	[CFU]	Student *t* test
wt-*Pg*	$1x10^8 \pm 3.3\times10^6$	1.5 h	$1.8\times10^7 \pm 2.2\times10^6$	$p = 0.005$	$1.4\times10^7 \pm 2.1\times10^6$	$p = 0.0001$
*Pg*ΔPPAD	$1\times10^8 \pm 4.3\times10^6$		$1.4\times10^7 \pm 2.1\times10^6$		$0.9\times10^7 \pm 1.4\times10^6$	
wt-*Pg*	$1\times10^8 \pm 5.0\times10^6$	3 h	$2.6\times10^7 \pm 2.2\times10^6$	$p = 0.0004$	$2.1\times10^7 \pm 1.5\times10^6$	$p = 6.3 \times 10^{-07}$
*Pg*ΔPPAD	$1\times10^8 \pm 6.3\times10^6$		$1.8\times10^7 \pm 2.2\times10^6$		$1.2\times10^7 \pm 0.7\times10^6$	
wt-*Pg*	$1\times10^8 \pm 6.6\times10^6$	6 h	$2.1\times10^7 \pm 1.8\times10^6$	$p = 0.0005$	$1.7\times10^7 \pm 2.0\times10^6$	$p = 0.003$
*Pg*ΔPPAD	$1\times10^8 \pm 6.6\times10^6$		$1.4\times10^7 \pm 2.1\times10^6$		$1.1\times10^7 \pm 1.2\times10^6$	