Inactivation of Epidermal Growth Factor by *Porphyromonas gingivalis* as a Potential Mechanism for Periodontal Tissue Damage

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Porphyromonas gingivalis is a Gram-negative bacterium associated with the development of periodontitis. The evolutionary success of this pathogen results directly from the presence of numerous virulence factors, including peptidylarginine deiminase (PPAD), an enzyme that converts arginine to citrulline in proteins and peptides. Such posttranslational modification is thought to affect the function of many different signaling molecules. Taking into account the importance of tissue remodeling and repair mechanisms for periodontal homeostasis, which are orchestrated by ligands of the epidermal growth factor receptor (EGFR), we investigated the ability of PPAD to distort cross talk between the epithelium and the epidermal growth factor (EGF) signaling pathway. We found that EGF preincubation with purified recombinant PPAD, or a wild-type strain of *P. gingivalis*, but not with a PPAD-deficient isogenic mutant, efficiently hindered the ability of the growth factor to stimulate epidermal cell proliferation and migration. In addition, PPAD abrogated EGFR-EGF interaction-dependent stimulation of expression of suppressor of cytokine signaling 3 and interferon regulatory factor 1. Biochemical analysis clearly showed that the PPAD-exerted effects on EGF activities were solely due to deimination of the C-terminal arginine. Interestingly, citrullination of two internal Arg residues with human endogenous peptidylarginine deiminases did not alter EGF function, arguing that the C-terminal arginine is essential for EGF biological activity. Cumulatively, these data suggest that the PPAD-activity-abrogating EGF function in gingival pockets may at least partially contribute to tissue damage and delayed healing within *P. gingivalis*-infected periodontia.

Tissue remodeling and wound healing are extremely complex processes, which include tightly synchronized cell proliferation, migration, and repopulation (1, 2). These responses are of paramount importance for the maintenance of homeostasis in the periodontium permanently exposed to mechanical stress, potentially damaging environmental factors and, importantly, colonization with pathogenic bacteria. During periods of active inflammation, tissues are exposed to a wide range of cytokines and growth factors released by resident tissue cells or immune cells to modulate healing processes in a coordinated manner (3–7). These signaling molecules play a major role in the normal periodontal tissue turnover, as well as in periodontal repair and regeneration during chronic inflammatory periodontal disease (8, 9). Most prominent among these cell-derived factors are ligands for the epidermal growth factor receptor (EGFR), members of the epidermal growth factor (EGF) family (10–13).

EGF is expressed as a pro-form, which is proteolytically processed into a biologically active peptide encompassing 53 amino acid residues. The binding of the processed peptide to the EGFR induces receptor homo- or heterodimerization and subsequent activation of a complex by autophosphorylation catalyzed by a tyrosine kinase domain of the EGFR molecule (14, 15). Both EGF and EGFR are expressed in periodontal tissues (16, 17). In healthy human gingiva, EGFR expression is limited to the gingival epithelium but during periodontal disease, the resultant tissue damage and the subsequent regeneration process induces a drastic increase in EGFR expression in the periodontal ligaments (7, 18, 19). This observation correlates with the finding that EGF-dependent signaling is involved in regulation of numerous biological pathways in the periodontium, including regulation of cell proliferation, migration, and differentiation (7, 16, 20).

Thus, the importance of EGF in periodontal health is highlighted by the fact that a single-nucleotide polymorphism within the EGF gene has been found to be associated with development of severe chronic periodontitis (21).

Periodontitis is a microbial biofilm-driven chronic inflammatory condition associated with the presence of specific bacterial pathogens, including *Porphyromonas gingivalis* (22–26). *P. gingivalis* is a Gram-negative, nonmotile, anaerobic asaccharolytic black-pigmented bacterium furnished with a wide range of virulence factors, including fimbriae, hemagglutinins, and numerous proteinases which are indispensable for the colonization, growth, and deterrence of antibacterial activity of the immune system (27). Except for these well-characterized pathogenicity traits, *P. gingivalis* also produces other enzymes recognized as potential virulence factors, which also may be involved in disease development and progression (28, 29). One of these poorly characterized enzymes is the *P. gingivalis* peptidylarginine deiminase (PPAD), which is able to modify proteins by deimination of the arginine residues, thereby converting them to citrulline (29, 30). Despite

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the low level of sequence similarity, all catalytic and guanidine-binding residues essential for peptidylarginine deiminase activity of eukaryotic enzymes are conserved in PPAD (31). In contrast to mammalian enzymes, PPAD is able to deiminate both free arginine and peptidylarginine, preferentially targeting an Arg residue at the carboxy terminus of a peptide/polypeptide chain (29). In contrast to peptidylarginine deiminase activity of PPAD, other known bacterial homologous enzymes can only deiminate free arginine or agmatine residues (arginine derivative) (31).

Conversion of positively charged arginine into neutral citrulline may affect biological function of a protein in a number of ways: (i) by impacting the folding and stability of a polypeptide chain, (ii) by altering susceptibility to proteolysis of the modified protein, or (iii) by abrogating the biological activity dependent on an exposed Arg residue(s). Indeed, it has been shown that endogenous PAD citrullination of histones (32, 33), chemokines (34), and bactericidal peptide LL-37 (35) affected gene expression (33, 36), inflammatory reaction (37, 38), and antimicrobial activity in the lungs (35, 39), respectively.

The presence of bacterial products, including *P. gingivalis* outer membrane vesicles (27) and gingipains (40) in gingival tissues distant to the bacterial plaque strongly argues that PPAD can also penetrate deeply into the connective tissue. Outer membrane-associated enzymes such as PPAD could disseminate into the tissues via outer membrane vesicles or diffusion of the soluble form and modify EGF within the inflamed periodontium. Therefore, we hypothesized that PPAD can inactivate EGF and negatively impacting on the course of periodontal tissue regeneration during the quiescence phase of periodontitis or after tooth debridement. Such contention is supported by well-documented observation of refractory to periodontal treatment disease due to the persistent presence of *P. gingivalis* (41–43).

The present study shows that EGF can be inactivated by PPAD and is the first report to describe modulation of a eukaryotic signaling molecule by a bacterial PAD enzyme. Biochemical analysis clearly demonstrated that PPAD citrullinates C-terminal arginine of EGF with subsequent impairment of EGF biological activity. The functional analysis of the EGF activity included evaluation of EGF-induced cell proliferation and migration. Furthermore, an assay showing that the induction of EGF-dependent SOCS3 (suppressor of cytokine signaling 3) and IRF-1 (interferon regulatory factor 1) gene expression was conducted. Surprisingly, only citrullination of the C-terminal arginine residue resulted in impairment of EGF function, since citrullination of internal residues with human PAD2 and PAD4 enzymes did not abrogate peptide function. Thus, the decreased activity of EGF in gingival pockets may at least partially contribute to the observed tissue damage and delayed healing within human periodontium during *P. gingivalis* infection.

MATERIALS AND METHODS

Cell culture. Human primary fibroblasts (44) were maintained in D10 medium (Dulbecco modified Eagle medium [DMEM; PAA Laboratories, Germany] supplemented with 10% heat-inactivated fetal bovine serum [PAA Laboratories], penicillin [100 U ml⁻¹], and streptomycin [100 µg ml⁻¹]). The cells were cultured on T-25 flasks (TPP, Switzerland) at 37°C with 5% CO₂. Cultures were routinely tested for the presence of *Mycoplasma* spp. and proved negative.

Bacterial culture. *P. gingivalis* strain W83 (45) and W83Δ*ppad* (46) were anaerobically grown in Schaedler broth supplemented with L-cysteine (0.05 g/ml), 1% dithiothreitol (DTT), menadione (0.5 mg ml⁻¹),

and hemin (1 mg ml⁻¹). *P. gingivalis* strains ATCC 33277 and ATCC 33277Δ*ppad* (46) were anaerobically grown in brain heart infusion (BHI) medium supplemented with hemin (5 µg ml⁻¹) and vitamin K (0.5 µg ml⁻¹). All cultures were placed in an anaerobic chamber MACS500 (Don Whitley Scientific, Ltd., Frederick, MD) in an atmosphere of 80% N₂, 10% CO₂, and 10% H₂. Bacteria from stocks stored at -80°C in storage medium (BHI supplemented with glycerol) were plated on blood agar, and a single colony was used to inoculate broth. The culture was grown anaerobically at 37°C until an optical density at 600 nm (OD₆₀₀) of 1.0 was reached.

Expression and purification of *P. gingivalis* PAD. Expression plasmid pET48b containing gene encoding *P. gingivalis* PPAD with a His₆ tag was kindly provided by Natalia Wegner (Kennedy Institute of Rheumatology, London, United Kingdom). Briefly, PPAD was expressed in *Escherichia coli* BL21(DE3)pLysS (Life Technologies, Poland) with a 3-h induction time in the presence of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 37°C. Protein was purified with a Ni-Sepharose 6 Fast Flow source (GE Healthcare Life Sciences, Germany) and Superdex 75 (GE Healthcare Life Sciences). The protein purity was evaluated with SDS-PAGE electrophoresis and N-terminal amino acid sequence analysis. The PPAD activity was assayed using a N_α-benzyloarginine ethyl ester (BAEE) as a substrate, and the activity was expressed in U/mol (1 U = 1 µmol of citrulline produced within the 1 h of the reaction) (47).

Influence of *P. gingivalis* on EGF activity. Cell proliferation assay was used to investigate *P. gingivalis* (strains W83 wild type [WT], W83Δ*ppad*, ATCC 33277 WT, and ATCC 33277Δ*ppad*) effect on EGF biological activity. Briefly, 400 ng of EGF was added to *P. gingivalis* cell culture adjusted to an OD₆₀₀ of 1.0 (total volume, 30 µl), followed by incubation for 3 h at 37°C in anaerobic conditions with shaking. Subsequently, the bacteria were removed by centrifugation (2,300 × g, 10 min), and the supernatant was used for further analyses.

Influence of purified human PAD2 and PAD4 and *P. gingivalis* PAD on EGF activity. In order to test whether incubation with purified PPAD affects EGF activity, 1 µg of EGF was incubated with 0.0126 U of PPAD, PAD2, or PAD4 (Modique Research) in dilution buffer (100 mM Tris, 10 mM CaCl₂, 2 mM L-cysteine [pH 7.5]) or with negative control samples (dilution buffer or 1× phosphate-buffered saline [PBS]) for 3 h at 37°C. After the incubation samples were diluted with D0 medium (DMEM supplemented with penicillin [100 U ml⁻¹] and streptomycin [100 µg ml⁻¹]) to reach the desired EGF concentration. Samples were used directly following the incubation.

Cell proliferation assay. Fibroblasts cultured as described above were seeded on 96-well plates (40,000 cells per well) in D10 medium. The cells were incubated for 24 h at 37°C with 5% CO₂. Subsequently, the cells were washed with 1× PBS and overlaid with 100 µl of fresh DMEM deprived of serum, supplemented with native EGF, bacteria, PAD2-, PAD4-, or PPAD-treated EGF, or dilution buffer. The cells were then cultured for 72 h as described above, and the medium was removed. Each well was washed with 1× PBS, and the cells were detached with 1× trypsin (0.5 mg/ml) supplemented with EDTA (0.22 mg/ml; PAA Laboratories). The cell suspension was mixed with trypan blue (1:1 [vol/vol]), and the cells were counted in a Fuchs-Rosenthal hemocytometer. In this way, the absolute number of cells was directly and precisely counted rather than using more indirect methods such as quantitative PCR and proteomics, which determine cell proliferation as a derivative of the nucleic acid or protein concentration.

Cell migration assay. Human fibroblasts cultured as described above were seeded on 12-well plates (500,000 cells/well) coated with collagen (Purecol; Nutacon, Netherlands) in D10 medium. The cells were incubated for 24 h at 37°C with 5% CO₂, reaching 100% confluence. Subsequently, the medium was removed, the cells were washed with 1× PBS, and native EGF, bacteria, and PPAD-treated EGF or control samples (dilution buffer) diluted in fresh D0 medium were added to each well. The medium was supplemented with mitomycin C (10 µg ml⁻¹) to eliminate cell proliferation, which may mask presentation of cell migration. To an-

analyze cell migration, a single linear scratch wound was made centrally across each cell monolayer using a pipette tip (48). Cell migration in the presence of untreated and treated EGF and in control samples was visualized at 24 h postinoculation using phase-contrast microscopy (Nikon Eclipse Ti).

Nucleic acid extraction, reverse transcription, and real-time PCRs. Total cellular RNA was extracted using TRIreagent (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 instructions, using 5 μ l of the previously extracted RNA.

PCR amplification was carried out in a total volume of 10 μ l with DreamTaq PCR master mix (Fermentas) in the presence of forward and reverse primers as follows: for the amplification of β -actin, Bact5 (5'-CCA CAC TGT GCC CAT CTA CG-3') and Bact3 (5'-AGG ATC TTC ATG AGG TAG TCA GTC AG-3') at 500 nM each; for the amplification of SOCS3, 5_SOCS3 (5'-AGA GCC TAT TAC ATC TAC TCC GGG-3') and 3_SOCS3 (5'-TTC CGA CAG AGA TGC TGA AGA GTG-3'); and for the amplification of IRF-1, 5_IRF1 (5'-AGA GCA AGG CCA AGA GGA AGT CAT-3') and 3_IRF1 (5'-ACT GTG TAG CTG CTG TGG TCA TCA-3') at 500 nM each and template DNA (1 μ l). The PCR cycling conditions included initial denaturation for 3 min at 95°C, followed by 27 cycles of 20 s at 95°C, 30 s at 56°C, and 40 s at 72°C and then 5 min at 72°C for the final elongation.

Semiquantitative real-time PCR amplification was conducted with 2 $\times$ SYBR green mix (Sigma-Aldrich, Poland). Reaction was carried out in a total volume of 10 μ l in the presence of forward and reverse primers (Bact5/Bact3, 5_SOCS3/3_SOCS3, and 5_IRF1/3_IRF1) and template DNA (1 μ l) with Rox as a reference dye. The PCR cycling conditions included initial denaturation for 5 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 56°C, and 45 s at 72°C. Real-time amplification was followed by melting-curve assessment to confirm product identity.

HPLC analysis. A total of 10 μ g of EGF was diluted in 100 μ l of 0.1 M Tris-HCl (pH 7.5) and 10 mM CaCl₂ freshly supplemented with L-Cys to 2 mM. Next, to maintain the molar ratio of EGF to the enzyme at similar levels during sample preparation for activity assay, 63 mU of PPAD was added to the EGF sample, followed by incubation for 3 h at 37°C in a final volume of 200 μ l. The reaction was stopped by the addition of trifluoroacetic acid (TFA) to a 0.5% final concentration. The sample was resolved by using a high-performance liquid chromatography (HPLC) system (AKTA Micro; GE Healthcare, Germany) and a μ RPC C₂-C₁₈ 4.6/100 ST reversed-phase column in a 10 to 80% acetonitrile gradient using the mobile phases A (0.1% TFA in water) and B (80% acetonitrile with 0.08% TFA in water). Elution was monitored with a spectrophotometer (λ = 215 nm), and the results were recorded. Eluting fractions were collected on the 96-well microplate using AKTA system Frac-950 fraction collector (GE Healthcare), and fractions from each HPLC peak were pooled together and vacuum dried using a SpeedVac system (Eppendorf, Poland).

LC-MS/MS. Reversed-phase HPLC fractions dried under vacuum using the SpeedVac system were resuspended in 0.1 M NH₄HCO₃. The samples were reduced and alkylated in solution using 10 mM DTT at 37°C for 30 min, followed by incubation with 55 mM iodoacetamide for 30 min at room temperature in the dark. Samples were subsequently digested overnight at 37°C with 0.02 mass equivalents of sequencing-grade modified trypsin (Promega, Germany). The digested samples were acidified using 100% formic acid and purified using C₁₈ RP STAGETips (Proxeon, Odense, Denmark). The eluate was vacuum-concentrated and resuspended in 0.1% TFA (MS eluent A). The samples were subsequently analyzed by using tandem mass spectrometry (MS/MS). All experiments were performed on an AB-Sciex TripleTOF 5600 mass spectrometer with a Nanospray III Ion source (ABSciex). The HPLC setup used in conjunction with the mass spectrometer consisted of a Proxeon Easy-nLC HPLC system operated using intelligent flow control. A 10-cm fused silica column (75 μ m [inner diameter], 360 μ m [outer diameter]) packed with 3- μ m

C₁₈ reversed-phase material (Thermo Scientific). Mobile phases consisted of 0.1% formic acid in water (phase A) (Sigma-Aldrich) and 90% acetonitrile in 0.1% formic acid in water (phase B). Next, 12 μ l of tryptic peptide sample was automatically loaded onto the column and rinsed for 5 min at a flow rate of 250 nl/min, followed by a 30- to 40-min gradient from 5% phase B to 35% phase B at a constant flow rate of 250 nl/min. MS analysis was performed with 35 to 50 scans per 2.8- to 3.8-s cycle with an MS accumulation time of 250 ms, MS/MS accumulation times of 100 ms, and a peptide fragmentation threshold of 150 arbitrary intensity units. Every fifth sample, a calibration was performed automatically, ensuring mass accuracies below 15 ppm. The generated data was manually reviewed by checking for the presence of precursor ions with predicted m/z values matching putative citrullinated EGF peptides. Subsequently, the associated MS/MS spectra were reviewed for fragment ions proving or disproving presence of citrulline. The resulting data were assessed using the Mascot search engine (v2.3.02; Matrix Science, London, United Kingdom) against the Swiss-Prot database (Human Database, October 2011). The search was performed with trypsin specificity (with one allowed missed cleavage), carbamidomethyl as a fixed modification, and the oxidation of methionine as a variable modification. The peptide mass tolerance was set to 20 ppm, and the fragment mass tolerance was set to 0.05 Da. The instrument setting was ESI-QUAD-TOF, which permits b-NH₄⁺, y-b-NH₄⁺, and γ -H₂O fragment ion types. The score threshold for peptides was suggested by Mascot at around 20 to 40 (P < 0.05).

Statistical analysis. All experiments were repeated at least three times, and the results are expressed as means $\pm$ the standard deviations (SD). To determine significance of obtained results, comparison between groups was made using Student t test. P values of <0.05 were considered significant.

RESULTS

***P. gingivalis* peptidylarginine deiminase (PPAD) is responsible for the abrogation of EGF's ability to stimulate cell proliferation.** During periodontitis, bacteria growing below the gum line actively modulate inflammatory and healing processes in periodontal and gingival tissues orchestrated by cytokines and growth factors. Among the latter, EGF is a potent modulator of cell cycle progression (49), and its inactivation by *P. gingivalis* may have a negative impact on the regeneration of damaged tissue. To investigate *P. gingivalis* effect on EGF activity, primary human fibroblasts were cultured in the presence of EGF, EGF preincubated with two *P. gingivalis* strains (W83 or ATCC 33277 differing genetically and phenotypically [50]), or control samples and subsequently, fibroblast cell numbers were counted. As depicted in Fig. 1A, incubation of EGF in the presence of wild-type bacteria (both W83 and ATCC 33277 strains) invariably led to the abolishment of its biological activity to stimulate cell proliferation.

Gingipains are among the most important virulence factors produced by *P. gingivalis*; these proteolytic enzymes target numerous host proteins and are essential for bacterial pathogenicity *in vivo* (51–53). Surprisingly, incubation of EGF with gingipain-null mutant W83 KRAB (*P. gingivalis* W83 Δ kgp/ Δ rgpA Δ rgpB) also obliterated EGF's ability to stimulate proliferation of fibroblasts (Fig. 1B). This finding showed that gingipains have no effect on EGF activity and indicated that *P. gingivalis* produces another factor responsible for inactivation of EGF.

The presence of the Arg residue at the C terminus of EGF, the preferential target for PPAD, suggested that conversion of this Arg residue into citrulline by PPAD may inactivate EGF activity. To experimentally verify this assumption, EGF was preincubated with recombinant PPAD, and the growth factor activity was assessed in a cell proliferation assay. As shown in

FIG 1 *P. gingivalis* interferes with EGF-mediated signaling. (A) Incubation of EGF with *P. gingivalis* (strains W83 and ATCC 33277) results in complete inactivation of EGF signaling pathway. (B) Incubation of EGF with a gingipain-deficient mutant W83 KRAB ($\Delta kgp/\Delta rgpA \Delta rgpB$) results in a significant decrease in EGF-mediated fibroblast proliferation. (C) Incubation of EGF with PPAD-deficient mutants of *P. gingivalis* (strains W83 dPPAD and ATCC 33277 dPPAD), in contrast to wild-type bacteria, does not result in decreased EGF-mediated fibroblast proliferation. (D) Purified PPAD, but not human PAD2 or PAD4, hampers EGF-mediated proliferation of fibroblasts. All results are presented as the percentage of nonstimulated sample (NC). The significance of the observed differences between samples and positive control samples was analyzed using the Student *t* test (ns, not significant; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$). All experiments were repeated three times, and the results are expressed as means $\pm$ the SD.

Fig. 1D, such treatment inhibited EGF's ability to stimulate the proliferation of fibroblasts, confirming our contention that PPAD is at least partially responsible for EGF inactivation by whole *P. gingivalis* cells. On the other hand, although incubation of EGF with human PAD2 and PAD4 resulted in citrullination of internal Arg residues within the peptide (data not shown), no decrease in EGF activity was observed (Fig. 1D). The unique ability of PPAD to negate EGF function was further studied using isogenic PPAD-null mutants in two different genetic backgrounds of *P. gingivalis*. In contrast to parental strains W83 and ATCC 33277 (Fig. 1A), the PPAD-null mutants had no effect on EGF activity (Fig. 1C), demonstrating that regardless of the *P. gingivalis* genetic background PPAD is the sole factor responsible for EGF inactivation.

PPAD inhibits EGF ability to stimulate cell migration. Apart from its cell proliferation-stimulating activity during tissue regeneration, EGF also enhances cell migration (2, 54–56). To test the effect of PPAD on this well-known EGF activity, primary human

fibroblasts were incubated with native EGF, EGF preincubated with *P. gingivalis* or purified recombinant PPAD, and pertinent control samples in the presence of a cytostatic agent (mitomycin C). Cell migration was analyzed by light microscopy. The results clearly show that preincubation of EGF with wild-type bacteria and PPAD, but not with PPAD-null strains, abolished EGF ability to stimulate cell migration (Fig. 2). Again, these data confirm that PPAD is the only *P. gingivalis*-produced factor affecting the biological activity of EGF.

PPAD abrogates EGF-induced stimulation of expression of SOCS3 and IRF-1. Interaction of EGF with EGFR triggers a cascade of events resulting in expression of numerous genes responsible for EGF mediated effect. Among these genes are SOCS3 and IRF-1, a STAT-regulated cytokine-inducible negative regulator of cytokine signaling and an activator of alpha and beta interferon transcription, respectively (57). To determine whether PPAD can also abrogate this direct biological effect of EGF ligation to its receptor, the cells were incubated

FIG 2 *P. gingivalis* hampers EGF-mediated cell migration. (A) Incubation of EGF with WT *P. gingivalis* results in the complete abolishment of EGF's effect on the migration of human fibroblasts, whereas *P. gingivalis* $\Delta ppad$ has no effect on EGF activity. (a) Untreated control; (b) control with *P. gingivalis* W83; (c) control with *P. gingivalis* $\Delta ppad$; (d) EGF-treated control; (e) EGF-treated cells with *P. gingivalis* W83; (f) EGF-treated cells with *P. gingivalis* $\Delta ppad$. (B) Incubation of EGF with purified PPAD results in the complete abolishment of EGF effect on migration of human fibroblasts. (a) Untreated control; (b) control with sample buffer; (c) control with PPAD; (d) EGF-treated cells; (e) EGF-treated cells with sample buffer; (f) EGF-treated cells with PPAD. Black arrows show the apparent size of linear scratch wound made centrally across each cell monolayer using a pipette tip. All experiments were repeated three times, and representative images are presented.

for 1 h with native and PPAD-pretreated EGF and the level of SOCS3 and IRF-1 mRNA was determined by quantitative reverse transcription-PCR. As expected, native EGF increased the expression of the two genes up to 7-fold over the background level, and this effect was significantly reduced by growth factor pretreatment with PPAD (Fig. 3).

Incubation of EGF with PPAD results in the citrullination of arginine. Considering the PPAD preference for C-terminal Arg residues (29), it is logical to assume that EGF C-terminal arginine will be modified (Arg₅₃). To verify this hypothesis, native and PPAD-treated EGF were resolved by reversed-phase HPLC. Incubation of EGF with PPAD resulted in the increased retention time of the modified EGF in concordance with increased hydrophobicity of protein due to the conversion of arginine to citrulline (Fig. 4). To confirm that, indeed, the

modification was due to PPAD-catalyzed deimination of the C-terminal arginine, tryptic digests of the purified, the deiminated form of EGF was subjected to LC-MS/MS analysis. The identity of the detected peptides was confirmed by a Mascot search. Two peptides with mass shifts corresponding to the citrullination of arginine (+1 Da) were clearly recognized as DLKWWL-Cit and CQYRDLKWWL-Cit peptides derived from the C terminus of the EGF molecule. To further confirm the presence of the citrulline, the collision-induced dissociation (CID) fragmentation ions for these two peptides were manually inspected and shown to contain citrulline as the C-terminal residue. The identification of the EGF-derived peptides bearing Cit₅₃ in PPAD-incubated samples, compared to respective controls clearly indicates that PPAD-catalyzed deimination of the C-terminal Arg₅₃ is the sole modification of the molecule (Fig. 5).

FIG 3 (A and B) Modulation of SOCS3 (A) or IRF-1 (B) in the presence of EGF and citrullinated EGF. All results are presented as the quantity relative to β -actin as a reference gene. The significance of observed differences between test sample and EGF-treated control sample (EGF) was analyzed with a Student *t* test (***, $P < 0.0005$). All experiments were repeated three times, and results are expressed as means $\pm$ the SD.

DISCUSSION

Periodontitis is the most prevalent infectious inflammatory disease of humankind. It is estimated that up to 30% of the adult population suffers from periodontitis and that ca. 8% of these cases will result in tooth loss (58, 59). Furthermore, a causative link between periodontal disease and numerous other conditions has been recognized, including rheumatoid arthritis, cardiovascular disease, and aspiration pneumonia (60–62).

The physiological role of PPAD in periodontal disease development and progression remains unclear. Numerous hypotheses have been raised, including the production of ammonia during deimination process enhances the survival of *P. gingivalis* within the periodontal pocket, as reported previously for arginine deiminases and agmatine deiminases (29). Ammonia neutralizes the acidic environment and optimizes pH-dependent function of gingipain and PPAD, inactivates hemagglutinins, promotes ATP production, and has negative effects on neutrophil function (29, 63). Furthermore, it can be speculated that PPAD acts as a virulence factor by generating citrullinated peptides, which may assist the bacterium in spreading and circumventing the humoral immune response (29).

It has been previously shown that citrullination mediated by eukaryotic PAD enzymes results in modification or abrogation of protein or peptide function, influencing immune responses and tissue remodeling. For example, eukaryotic PAD-mediated citrul-

lination of various signaling molecules, including CXCL-5, CXCL-8, CXCL-10, CXCL-11, CXCL-12, and ING4 results in modulation of their activity and most likely is important for their biological functions *in vivo* (34, 37, 64–66). Surprisingly, no literature is available regarding bacterial PPAD modification of specific host functional protein(s). Here, we describe one of the potential effects of this enzyme on the host homeostasis.

EGF together with its receptor (EGFR) functions in a wide range of cellular processes, including cell fate determination, proliferation, migration, and apoptosis. In gingival epithelium, enhanced cell proliferation and migration triggered by EGF is associated with the turnover, repair, and regeneration of periodontal tissues (7, 16, 20). The ubiquitously expressed EGFR is a pleiotropic signal transducer, and its EGF-dependent activation triggers major signaling cascades such as, for instance, the Ras/mitogen-activated protein kinase (MAPK) or the MAPK pathway. Activation of these cascades recruits the SOS guanine nucleotide exchange factor to the plasma membrane. Subsequent exchange of the GTP for GDP on the small protein Ras leads to cell proliferation. Another fundamental process in tissue regeneration—enhanced cell motility—is regulated by EGFR-dependent phosphorylation of phospholipase C γ (PLC γ). Phosphorylated PLC γ catalyzes the formation of two important signaling molecules: inositol triphosphate and diacylglycerol. These transmitters stimulate the release of calcium ions from the smooth endoplasmic reticulum and activate protein kinase C, respectively, thus further contributing to the pleiotropic biological effect of EGF (67, 68).

EGF is known to be present in saliva, gingival tissues, and gingival crevicular fluid (GCF), contributing to the maintenance of tissue homeostasis. Previous studies on the levels of EGF in GCF are contradictory. Although some researchers have reported no marked modulation of the EGF levels (69), others have observed significant differences in EGF concentrations in GCF from deep and shallow sites in patients with periodontal disease (16). However, studies have shown that during inflammation associated with the development of periodontitis, the expression of EGFR is significantly increased, suggesting enhanced sensitivity of gingival tissues to EGF signaling (16). Further, EGF was shown to stimulate the proliferation of human periodontal ligament fibroblasts and human gingival fibroblasts (70, 71), apparently through the mechanisms described above.

It has been previously shown that *P. gingivalis* lipopolysaccharide (LPS) modulates the regenerative effect of EGF via downregulation of EGFR-dependent signaling (72). It was suggested that this phenomenon is directly related to the fact that both epidermal growth factor and LPS activate the MAPKs to modulate cell proliferation, cell survival, and the release of inflammatory mediators. The observed alterations in EGF signaling caused by *P. gingivalis* LPS may be mediated by an array of events, including, among other possibilities, the differential recruitment and altered kinetics of activation of upstream mediators in response to LPS and EGF (72). The effect observed here cannot be associated with LPS activity, since no decrease in EGF activity following incubation with LPS-positive ATCC 33277 Δ ppad or W83 Δ ppad *P. gingivalis* mutants was observed. One may therefore assume that the effect observed in the present study is complementary to previously observed phenomena and together may lead to severe tissue damage and remodeling.

It is surprising that gingipains, which are considered important virulence factors of *P. gingivalis* (73, 74), did not appear to show

FIG 4 Citrullination of EGF by *P. gingivalis* PPAD. A total of 10 μ g of EGF (black line), EGF in the reaction buffer (faint gray line), or EGF with PPAD (dark gray line) was resolved using reversed-phase HPLC. The eluted fractions were collected and, after trypsinization, analyzed by LC-MS/MS (see Fig. 5).

any prominent effect on EGF *in vivo*, since incubation with the *P. gingivalis* $\Delta ppad$ strain equipped with a whole set of gingipains did not lead to decrease in EGF activity and incubation with the *P. gingivalis* W83 gingipain-null mutant ($\Delta kgp/\Delta rgpA \Delta rgpB$) led to the complete abolition of EGF activity. Furthermore, incubation with *P. gingivalis* $\Delta ppad$ strains expressing the whole set of gingipains did not result in a significant degradation of EGF. In contrast, HPLC analysis of PPAD-treated EGF revealed a significant shift of the retention time, suggesting an altered peptide charge and increased hydrophobicity. To further confirm the observation, subsequent analysis by MS was performed. The sole detected modification of the peptide was citrullination of the C-terminal Arg₅₃. The lack of detectable citrullination of internal arginines is consistent with previous observations that PPAD is specific mostly toward the C-terminal residues, and its ability to modify other amino acids is questionable and most likely minimal (29). Furthermore, citrullination of internal EGF Arg residues (Arg₄₁ and Arg₄₅) with human PADs did not result in decreased EGF activity, which provides further evidence that modification of C-terminal Arg is sufficient to hamper EGF activity. As mentioned above, this observation is rather surprising, since it was previously reported that excision of the EGF C-terminal Arg₅₃ residue is not associated with a loss of function (75). Nonetheless, one may assume that modification of the molecule charge may affect the ability of the peptide to interact with its receptor. Comparison of the EGF structure in complex with its receptor, as de-

termined by using X-ray diffraction (76) or nuclear magnetic resonance (77), reveals the unfolded organization of the C-terminal α -helix, allowing the essential Leu₄₇ residue to be exposed and available for interaction with site 3 of the EGFR. This is in agreement with the “hand-glove” model of receptor ligand interaction. Based on the described mechanisms, it is hypothesized that citrullination of C-terminal arginine introduces additional hydrophobic interactions, which stabilize the C-terminal helix and prevent structural changes required for the receptor binding. Such a process would likely result in inactivation of the molecule. The proposed mechanism, termed “subtraction by addition” explains both the observed redundancy of C-terminal Arg₅₃ for receptor binding (75) and the deamination-mediated inactivation of EGF. Although intriguing, since it would provide the structure-function link for EGF inactivation, the proposed mechanism requires structural verification, which is beyond the scope of the present study.

We demonstrate here for the first time the direct effect of PPAD on a eukaryotic signaling molecule, showing that this bacterial enzyme is not only active as a source of ammonia but may also modulate the local microenvironment. EGF is one of the essential factors in wound healing and tissue regeneration, and its inactivation may impair regeneration and/or healing of the periodontal tissues. The PPAD-induced disruption of cross talk between epithelium and the EGF signaling pathway may have pronounced consequences for disease progression (78). Nonetheless,

FIG 5 (A) PPAD modifies C-terminal Arg₅₃ in mature EGF MS/MS collision-induced dissociation (CID) fragmentation spectra of the precursor ion 573.8 detected for EGF treated with PPAD. The spectrum matches the peptide DLKWWEL[Cit]. The y1 ion mass of 176.1 matches the expected mass of a C-terminal citrulline residue. The series b and y ions are listed above the spectra, and the ions providing additional evidence are listed where appropriate; an asterisk (*) denotes ions generated by loss of ammonia. A superscript zero indicates loss of water, and the “++” symbol denotes double-charged ions. (B) Summary of modification sites detected using CID MS. The sites were confirmed by review of the CID fragmentation spectra verifying whether peak patterns are best explained by citrullination. The listed y ions (C-terminal fragments) and b ions (N-terminal fragments) show the observed evidence ions and/or peaks confirming citrullination. Nine *m/z* variants of the four peptides were reviewed for all samples, but only results for peptides found to be potentially citrullinated are shown. All of the identified peptides had mass deviations below 12 ppm, and the average deviation was 3.7 ppm. Peptide matches indicate the number of EGF peptide spectra recorded for the sample, and sequence coverage indicates the percentage of the EGF sequence covered by the detected peptides.

further clinical studies are required to determine the validity of such a hypothesis and its consequence(s) in the treatment of periodontitis.

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