Peptidyl Arginine Deiminase from Porphyromonas gingivalis Abolishes Anaphylatoxin C5a Activity*

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Background: Pathogenic bacteria avoid killing by phagocytes through inhibition of C5a chemotactic activity.

Results: Periodontopathogen Porphyromonas gingivalis expresses unique peptidylarginine deiminase, which inactivates C5a by converting C-terminal arginine to citrulline.

Conclusion: Citrullination of C5a constitutes a novel virulence strategy that may contribute to immune evasion by P. gingivalis.

Significance: P. gingivalis peptidylarginine deiminase is a potential target for drug development.

Evasion of killing by the complement system, a crucial part of innate immunity, is a key evolutionary strategy of many human pathogens. A major etiological agent of chronic periodontitis, the Gram-negative bacterium Porphyromonas gingivalis, produces a vast arsenal of virulence factors that compromise human defense mechanisms. One of these is peptidylarginine deiminase (PPAD), an enzyme unique to P. gingivalis among bacteria, which converts Arg residues in polypeptide chains into citrulline. Here, we report that PPAD citrullination of a critical C-terminal arginine of the anaphylatoxin C5a disabled the protein function. Treatment of C5a with PPAD in vitro resulted in decreased chemotaxis of human neutrophils and diminished calcium signaling in monocyte cell line U937 transfected with the C5a receptor (C5aR) and loaded with a fluorescent intracellular calcium probe: Fura-2 AM. Moreover, a low degree of citrullination of internal arginine residues by PPAD was also detected using mass spectrometry. Further, after treatment of C5 with outer membrane vesicles naturally shed by P. gingivalis, we observed generation of C5a totally citrullinated at the C-terminal Arg-74 residue (Arg74Cit). In stark contrast, only native C5a was detected after treatment with PPAD-null outer membrane vesicles. Our study suggests reduced antibacterial and proinflammatory capacity of citrullinated C5a, achieved via lower level of chemotactic potential of the modified molecule, and weaker cell activation. In the context of previous studies, which showed crossstalk between C5aR and Toll-like receptors, as well as enhanced arthritis development in mice infected with PPAD-expressing P. gingivalis, our findings support a crucial role of PPAD in the virulence of P. gingivalis.

Porphyromonas gingivalis is a major causative agent of periodontitis, a chronic inflammatory disease of tooth-supporting structures that affects up to 30% of the world’s population (1). This Gram-negative, anaerobic bacterium uses a large arsenal of virulence factors such as hemagglutinins/adhesins, fimbriae, and proteolytic enzymes to facilitate colonization of the gingival sulcus, to generate nutrients and growth factors, and to provide protection from the host immune system (2). The latter is achieved by sophisticated manipulation of the host inflammatory response through activation of coagulation factors and contact activation systems, corrupting complement functions, shedding receptors, and modifying cytokines and intracellular signaling (3). In this way, P. gingivalis maintains the local chronic inflammatory reaction and thrives in this environment to access host components essential for bacterial growth.

Recently, P. gingivalis peptidylarginine deiminase (PPAD), an enzyme absent in other prokaryotes, which converts Arg residues in polypeptide chains into citrulline (Cit), has been hypothesized to be a potential virulence factor (4). Post-translational modification mediated by the deiminase activity of PPAD may change protein function in a similar manner to that described for citrullination of chemokines (5) and antibacterial peptide LL-37 (6) by endogenous PADs. However, in stark contrast to mammalian PADs, PPAD has a strong preference for C-terminal Arg residues, probably to neutralize the positive charge at the C-terminus of (poly)peptide fragments generated.

The abbreviations used are: PPAD, peptidylarginine deiminase; C5aR, C5a receptor; Cit, citrulline; Rgp, arginine-specific gingipain; OMV, outer membrane vesicle; Bis-Tris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)-propane-1,3-diol; nLC-MS/MS, nano-LC-MS/MS.

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by degradation of proteins by \textit{P. gingivalis} Arg-specific gingipains (7). Furthermore, PPAD can also abrogate essential biological activities of host proteins and peptides, which are dependent on the C-terminal Arg residues. This hypothesis is supported by the finding that C-terminal citrullination of EGF by PPAD impaired biological activity of EGF (8).

Anaphylatoxin C5a is a polypeptide of 74 residues released from C5 by C5vertase during complement activation. Widespread expression of two C5a receptors (C5aR and C5L2) throughout the body ensures a variety of biological responses, including chemotaxis of inflammatory cells, phagocytosis, respiratory burst, vascular permeability, and releases of pro-inflammatory cytokines and chemokines. The C-terminal Arg residue is crucial for C5a function, and \textit{in vivo}, the molecule is rapidly converted by carboxypeptidases to the far less potent C5a-desArg of significantly lower affinity for C5aR (9). Because C5a is an essential component of the inflammatory response to bacterial infection, it was of interest to determine whether PPAD can abolish its biological activity. Here, we showed that PPAD efficiently deiminated C-terminal Arg in C5a \textit{in vitro} and that citrullinated Arg-74 of C5a can be found in C5 samples treated with outer membrane vesicles isolated from \textit{P. gingivalis} culture. Moreover, this modification decreased the ability of C5a to induce calcium influx in a monocytic cell line expressing C5aR and strongly reduced its chemotactic potential for neutrophils.

**EXPERIMENTAL PROCEDURES**

**Ethics Statement**—The regional ethical board in Lund has approved collection of blood from healthy volunteers after informed consent.

**Proteins**—As a member of the C-terminal domain protein family of \textit{P. gingivalis}, native PPAD was engineered to be secreted from a mutant of \textit{P. gingivalis} W83 as a soluble form with a hexahistidine affinity tag using the same molecular strategy as was reported previously for the RgpB protease in the mutant 662i6H (10). Subsequently, PPAD was purified from the culture medium via ion-exchange and gel filtration chromatography. Briefly, the bacteria were cultured in enriched tryptophan broth medium for 72 h, cells were removed by centrifugation, and proteins in cell-free culture medium were precipitated with acetone, resuspended in phosphate buffer (pH 6.5), dialyzed, and passed through a DE-52 column (Whatman) to remove excess hemin. The flow-through was dialyzed against 50 mM Tris-Cl, 0.02% NaN3, pH 8.0, and loaded on a Mono Q column (GE Healthcare). Adsorbed proteins were eluted with NaCl gradient, and fractions containing PPAD activity were pooled. The final PPAD purification was achieved by gel filtration chromatography using Superdex 75 column (GE Healthcare). The purity of PPAD was evaluated by SDS-PAGE followed by silver staining. Activity of PPAD was tested using a colorimetric assay as reported previously (11). Before any assay, PPAD was preactivated by incubation for 20 min 37 °C in 10 mM Tris, pH 8.0, buffer in presence of 1 mM L-cysteine. Because a very minor contamination (less than 0.5%) with arginine-specific gingipain (Rgp) was observed in some batches of purified PPAD, assay buffers were supplemented with 1 mM KYT-1, a specific Rgp inhibitor (Peptide International). At the concentrations used, KYT-1 and l-cysteine did not influence the assays (data not shown). C5a and C5a-desArg were purchased from Complement Technology.

**Isolation of Outer Membrane Vesicles (OMV) from \textit{P. gingivalis} Strains—**\textit{P. gingivalis} strains W83 and mutant \textit{Δppad} (in W83 background) (12) were cultivated in enriched Schaedler broth (supplemented with 5 mg/ml hemin, 0.5 mg/ml menadione, and 5 mM L-cysteine, and for mutant strain, supplemented with 5 µg/ml erythromycin) overnight in anaerobic conditions to reach optical density = 1. An aliquot (100 ml) of each culture was gently sonicated in a sonicator bath to release OMV to the cell supernatant. Bacteria were removed by centrifugation (20 min, 10,000 x g), and the remaining supernatant from each strain was subjected to ultracentrifugation (1 h, 150,000 x g, 4 °C). Pellets containing OMV fraction were resuspended in 20 mM Bis-Tris, pH 6.8, 150 mM NaCl, 5 mM CaCl2 and analyzed for PPAD- and Arg-specific gingipain (Rgp) activity. Rgp activity was tested using a spectrophotometric assay with \textit{Na}-benzoyl-L-arginine 4-nitroanilide hydrochloride as a substrate (13), and Rgp concentration was calculated based on the activity of the purified enzyme.

**HPLC Analysis of Full-length Form of C5a—**Samples (100 µl) containing 10 µg of C5a or C5a were treated with PPAD (in buffer containing 100 mM Tris, 5 mM L-cysteine, 5 µM KYT-1, pH 7.6) for 3 h at 37 °C. Samples were subsequently acidified with TFA (Sigma) and separated through a 4.6 × 150-mm Phenomenex Aeris C18 Widepore (Phenomenex) on AKTamicro (GE Healthcare). Peptides were then eluted in H2O/0.1% TFA (A) and 80% acetonitrile/0.08% TFA (B) gradient and monitored at 215 nm.

**Deglycosylation and Proteolytic Treatment—**Samples of C5a and C5a-Cit prepared in the same way as for HPLC analysis were lyophilized and dissolved in 20 mM Tris, 100 mM NaCl, pH 8.0, containing 5 mM DTT and denatured at 95 °C for 1 h. To remove N-linked glycosylation, samples were treated with 0.25 units of N-glycosidase F (Sigma) per µg of C5a and incubated at 37 °C for 3 h. To generate two different variants of the C-terminal peptide, the deglycosylated sample was treated with either trypsin (1:25 ratio) or clostripain (4 units/µg of C5a) (both from Sigma) in the presence of 1 mM calcium acetate and incubated at 37 °C for 16 h. To uniformly modify all cysteine residues, samples were reduced by 5 mM DTT for 30 min at 25 °C followed by 30 min with 15 mM iodoacetamide. The samples were micropurified on StageTips (Thermo Scientific) according to the manufacturer’s instructions.

**Modification of C5 by Enzymes Present in OMVs from Different Strains of \textit{P. gingivalis}—**Purified C5 (Complement Technology) was mixed at 25:1 molar ratio to Rgp with OMVs isolated from \textit{P. gingivalis} strains W83 and \textit{Δppad} (in PBS buffer, pH 7.4, supplemented with 5 mM L-cysteine). Samples were incubated for 30 min 37 °C, and the reaction was stopped by the addition of TFA. Citrullination of the C-terminal Arg residue in C5a was assessed by mass spectrometry.

**Mass Spectrometry—**nLC-MS/MS analyses were performed on an EASY-nLC II system (Thermo Scientific) connected to a TripleTOF 5600+ mass spectrometer (AB SCIEX) equipped with a NanoSpray III source (AB SCIEX) operated under Analyst TF 1.5.1 control. The lyophilized samples were suspended.
in 0.1% formic acid, injected, trapped, and desalted isocratically on a Biosphere C18 column (5 μm, 2 cm × 100-μm inner diameter; Nano Separations). Peptides were eluted using 250 nL/min and a 20-min (for C5 samples treated with OMVs) or 50-min (for C5a samples treated with purified PPAD) gradient from 5 to 35% mobile phase B (0.1% formic acid and 90% acetonitrile). Eluted peptide samples were separated on a 15-cm analytical column (75-μm inner diameter) with RP ReproSil-Pur C18-AQ 3-μm resin (Dr. Maisch GmbH). The collected MS files were converted to Mascot generic format (MGF) using the AB SCIEX MS Data Converter beta 1.1 (AB SCIEX) and the "protein pilot MGF" parameters. The generated peak lists were searched against an in-house database containing the C5a sequence using the Mascot search engine (Matrix Science). Search parameters were either trypsin or Arg-C as protease allowing three missed cleavage sites. Carbamidomethyl was set as fixed modification, and citrullination of Arg, deamidation of Asn or Gln, and oxidation of Met residues were set as variable modifications. Peptide tolerance and MS/MS tolerance were set to 10 ppm and 0.2 Da, respectively.

**Cell Culture Conditions**—U937 cells expressing C5a receptor (U937-C5aR) were maintained in RPMI medium containing 10% FCS and G418 (400 μg/ml) at 37 °C in a humidified 5% CO2 atmosphere (14).

**Calcium Mobilization Assay**—U937-C5aR cells were harvested by centrifugation, washed twice with PBS, resuspended in HBSS (0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na2HPO4, 0.44 mM KH2PO4, 1.0 mM MgSO4, 4.2 mM NaHCO3, 1 g/liter glucose) with 1.3 mM Ca2+ at 2 × 106 cells/ml and incubated in the presence of a cell-permeable fluorescent intracellular calcium probe (Fura-2 AM, Invitrogen) for 30 min 37 °C with gentle agitation. After double washing with HBSS/Ca2+, cells were resuspended in the same buffer at 1.25 × 106 cells/ml and kept on ice until use. Cell response to a test ligand was monitored with continuous fluorescence measurements at λexcitation = 340 and 380 nm and λemission = 510 nm, using the Infinite 200 PRO microplate reader (Tecan) and i-Control software. After a few seconds of the basal signal measurement, C5a (50 μL, final concentration 10 nM) or C5a pretreated for 1 h at 37 °C with PPAD was mixed with 200 μL of cell suspension, and measurement was continued for 5 min. Values of fluorescence intensity for each time point were recalculated as the ratio of fluorescence intensity: λemission/λexcitation = 340 nm/λemission = 510 nm at λexcitation = 380 nm. As controls, C5a-desArg and C5a-desArg preincubated with PPAD were used.

**Neutrophil Chemotaxis**—Human neutrophils were isolated from healthy donors with Histopaque-1119/Percoll (15), washed twice, resuspended in PBS supplemented with 0.5% human serum albumin (Sigma-Aldrich) at 106 cells/ml, and labeled with 1 μM carboxyfluorescein succinimidyl ester (Fluka) for 15 min at room temperature with gentle agitation. After staining, cells were washed twice and resuspended in PBS with 0.5% human serum albumin and 4% heat-inactivated (30 min, 56 °C) hirudin-treated human plasma at a concentration of 5 × 106 cells/ml prior to use. The purity of neutrophil population (defined as CD14 low/CD15+/CD16−) was determined with a CyFlow Space (Partec) flow cytometry system using α-CD14-PE (IgG2a) (BD Biosciences) and α-CD15-FITC (IgM) and α-CD16-APC (IgG1) (ImmunoTools) antibodies and confirmed as 90–95%.

Migration of neutrophils was assessed using a disposable 96-well cell migration system with 3-μm polycarbonate membranes (ChemoTx; Neuro Probe). C5a was preincubated with serial dilutions of PPAD for 1 h at 37 °C. The samples were then supplemented with heat-inactivated human plasma at the same concentration as for neutrophil medium and applied to the wells of Chemotx plate. C5a at a final concentration of 12.5 nM was used as a positive control, whereas 125 nM C5a-desArg, treated and untreated with PPAD, served as a negative control. Neutrophil suspension (50 μL) was then applied to each well of the upper filter, and the plate was incubated for 60 min 37 °C in humidified air with 5% CO2. The membrane filter was then removed, and the cell suspensions from the bottom wells were transferred to a flat-bottom, 98-well black plate (Nunc). The bottom wells of the Chemotx plate were washed twice with 30 μL of PBS, and the washes were pooled with the corresponding cell suspensions from the bottom wells. Fluorescence intensity signals from cells were measured for 0.1 s using a Wallac Victor 1420 multilabel counter (PerkinElmer) using λexcitation = 485 nm and λemission = 535 nm.

**Statistical Analysis**—One-way analysis of variance with Dunnett’s post test was used to estimate whether the observed differences between groups were statistically significant. Data were analyzed using GraphPad Prism 5.0.

**RESULTS**

**PPAD Citrullinates C5a**—The purity of PPAD was determined with SDS-PAGE followed by silver staining (Fig. 1A). To confirm citrullination of C5a by PPAD, native and PPAD-treated C5a were analyzed using HPLC. C5a in the native form eluted in two peaks from the C-18 column, whereas PPAD-treated C5a eluted in four peaks, at retention times different from those of the native peptide. This indicated citrullination of arginine residue(s) in C5a by PPAD (Fig. 1B). To determine which Arg residue(s) were deaminated, the native and PPAD-treated C5a were digested with trypsin or clostripain and subsequently analyzed by mass spectrometry on-line with RP-HPLC. Among tryptic peptides derived from PPAD-incubated C5a, one abundant peptide showed a clear shift in retention time as compared with the control C5a-derived peptides. Similarly, a single peptide with shifted retention time was also found in clostripain-digested, PPAD-treated C5a versus native C5a (data not shown). Subsequently, the peptides were identified by MS/MS as derived from the C terminus of C5a (DMQLGR and ANISHKDMQLGR). The citrullination of terminal Arg residue in these peptides was indicated by a 1-Da mass shift, peptide score (sequence information from MS), as well as the retention time shifts (Fig. 1C). In PPAD-treated C5a, only the citrullinated version of the C terminus was detected, indicating complete modification at the C terminus. Native C5a did not exhibit significant amounts of this modification. Interestingly, we confirmed endoarginine deaminase activity of PPAD by detecting modifications of positions 40 (AAR15LGPR) and 46 (ISLGPR15CIK) in the tryptic digest of PPAD-treated C5a. These, however, were much less abundant than the C-terminal modification.
OMVs Released from P. gingivalis Generate Citrullinated form of C5a from C5—Based on previous studies showing the release of active C5a from C5 by Arg-specific gingipains (16) and the presence of both Rgp and PPAD in OMVs from P. gingivalis (17, 18) we incubated intact C5 with OMVs from the wild-type P. gingivalis and the isogenic mutant strain lacking
PPAD. Mass spectrometry analysis revealed full citrullination of the residue Arg-74 of C5a in C5 samples treated with wild-type strain OMVs. No such modification was present in the C5 sample treated with OMVs derived from Δppad. In this sample, only the native C5a C terminus was detected (Fig. 1D). Significantly, the gingipain activity in wild-type and PPAD-null vesicles was identical, and both degraded C5 to the same extent, releasing C5a. Nevertheless, citrullination of generated C5a was observed only after treatment with OMVs from wild-type P. gingivalis. This clearly indicates that PPAD very efficiently modifies C5a released by gingipains.

Citrullinated C5a Has Decreased Chemotactic Activity—C-terminal arginine of C5a anaphylatoxin is crucial for biological activity of this peptide. We hypothesized that deimination of this residue by PPAD, resulting in generation of neutral citrulline, should suppress proinflammatory activity of C5a, in a manner similar to physiological removal of C-terminal arginine by carboxypeptidases. Indeed, preincubation of C5a with PPAD strongly reduced its chemotactic activity for neutrophils, in a concentration-dependent manner, whereas it had no effect on the activity of C5a-desArg (Fig. 2A). This result suggests that C5a-Cit, similar to C5a-desArg, has significantly lower affinity for C5aR on neutrophils. In keeping with this finding, treatment of C5a with PPAD also impaired its ability to induce calcium influx in a myeloid-derived cell line transfected with C5aR (Fig. 2, B and C). Interestingly, at higher concentrations and/or prolonged incubation with PPAD, the enzyme totally abrogated the capacity of C5a to activate neutrophils and U937 C5aR cells. In contrast, C5a-desArg treated with PPAD did not show altered potential to stimulate neutrophil chemotaxis and calcium release in U937-C5aR cells. As expected, native C5a-desArg had much lower activity than C5a in these assays.

**DISCUSSION**

The complement system constitutes an essential part of innate immunity. Its activation, in a cascade-like manner, unleashes a spectrum of molecules aimed at destroying invading microbes. The membrane attack complex perforates cell membranes, anaphylatoxins C3a and C5a attract and activate neutrophils, opsonization with fragments of C3b facilitates phagocytosis and intracellular killing, and finally, activated components of complement enhance adaptive immune response. Therefore, it is not surprising that successful human pathogens developed a variety of mechanisms to inhibit complement activation and/or to neutralize activated components. Several of the latter strategies target C5a or C5aR. Proteolytic inactivation of C5a by Serratia marcescens 56K protease, protease ScpA of Streptococcus pyogenes, and ScpA-like enzymes of group B streptococci results in inhibition of C5a-mediated proinflammatory and chemotactic signaling that slows the influx of antigens.

![FIGURE 2. PPAD citrullinates C5a, decreasing its chemotactic ability against neutrophils and calcium influx into U937 C5aR cells.](image-url)
inflammatory cells and hinders removal of bacteria from the initial site of invasion (19). The importance of ScpA as a specialized virulence factor is underscored by the narrow specificity of this protease. The enzyme has no activity against intact C5 or other proteins but cleaves exclusively C5a at the C terminus, removing part of the region that interacts with C5aR on neutrophils (20).

In contrast to other bacterial pathogens for which suppression of the inflammatory response is the immune evasion strategy, the key periodontal pathogen *P. gingivalis* seems to thrive in the inflammatory milieu. Through proteolytic degradation of C3 and C5 (21) and hijacking complement regulator C4BP (22), the bacterium efficiently blocks assembly of membrane attack complex and opsonization. However, the cleavage of C5 by gingipains is associated with the release of fully functional C5a (16). The release of this potent chemoattractant and activator of neutrophils has limited effects on *P. gingivalis*, which is fairly resistant to killing by granulocytes and macrophages. It has been reported that in murine macrophages, C5a induces a crosstalk between C5aR-dependent signaling and activated Toll-like receptor-2 (TLR-2) resulting in *P. gingivalis* survival in vivo (23). The importance of C5a signaling for *P. gingivalis* survival was verified by the finding that specific blockade of C5aR enabled eradication of infection (24). This mechanism described for murine macrophages and the murine model of periodontitis is at odds with two countermeasures performed, reconciles PPAD as an important virulence factor contributing to *P. gingivalis* survival in vivo.

Modification of C5a by PPAD may generate a potential virulence factor due to the presence of C-terminal citrulline. Together with other C-terminally citrullinated peptides derived from bacterial and host proteins, including those derived from Rgps-degraded fibrinogen and enolase, C5a-Cit adds to the burden of post-translationally modified antigens. Occurring within a chronically inflamed periodontal tissue, these citrullinated epitopes may initiate breakdown of immune tolerance against host citrullinated proteins and the generation of autoantibodies in susceptible individuals, eventually leading to symptoms associated with rheumatoid arthritis (28). This contention is supported by the finding that collagen-induced arthritis was exacerbated in mice infected with wild-type *P. gingivalis* as manifested by earlier onset, accelerated progression, and enhanced severity of the disease, including significantly increased bone and cartilage destruction (12). The ability of *P. gingivalis* to augment arthritis was dependent on the expression of PPAD and associated with increased levels of citrullinated epitopes.

Taken together, in this study, we described a novel pathogenic strategy of bacterial pathogen to inactivate the antibacterial, proinflammatory activity of C5a by deamination of its C-terminal arginine. Such an approach is, thus far, unique for *P. gingivalis*, which is the sole bacterium able to express peptidyl arginine deiminase, with strong preference for C-terminal arginine. In this context, PPAD emerges as an important virulence factor, which warrants further investigation.

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