Porphyromonas gingivalis (Gingipains) from the periodontal pathogen, Porphyromonas gingivalis

IMPLICATIONS OF IMMUNE EVASION∗

(Received for publication, December 16, 1997, and in revised form, January 17, 1998)

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Porphyromonas gingivalis is one of the major pathogens associated with adult periodontitis, a major chronic inflammatory disease. Potent proteinases elaborated by these bacteria aid directly and indirectly in both the development of the pathophysiology of the disease and in host defense evasion. For these reasons they are considered key virulence factors. To investigate whether possible immune evasion mechanisms involve the dysregulation of the host cytokine network, we examined the ability of P. gingivalis cysteine proteinases, including Arg-specific gingipains HRGP and RGP2 and Lys-specific KGP, to degrade the proinflammatory cytokine tumor necrosis factor-α (TNF-α). All three gingipains rapidly degraded TNF-α as exhibited by immuno blot analysis. Moreover, all biological activity was significantly reduced over extended incubation periods with the proteinases tested, whereas the host neutrophil proteinases were ineffective. These results indicate that the gingipain proteinases elaborated by P. gingivalis are capable of disrupting the cytokine network at the site of infection through the degradation of the proinflammatory cytokine TNF-α, suggesting the removal of one of several mediators important to the function of polymorphonuclear leukocytes. Such a mechanism is likely to be utilized by other infective organisms not only for survival but also for growth and proliferation.

Adult periodontitis occurs as a result of a bacterial infection that, if left untreated, may result in tooth loss (1, 2). It is commonly characterized as a chronic inflammatory disease, exemplified by inflamed gingival tissues, increased crevicular fluid, and massive polymorphonuclear leukocyte (PMN)1 infiltration of the gingival tissues, all of which contribute to both connective tissue loss and alveolar bone loss (3, 4). The development of adult periodontitis is now believed to be a result of the uncontrolled activity of two members of a family of cysteine proteinases synthesized and secreted by the organism Porphyromonas gingivalis, an opportunistic pathogen that colonizes beneath the gum line in infected individuals. The two proteinase types are referred to as Arg-gingipains (HRGP and RGP2) and Lys-gingipain (KGP) because of their specificity for cleavage after arginyl and lysyl residues, respectively. HRGP differs from RGP2 in that the protein contains an additional adhesion domain accounting for its higher molecular mass in comparison to RGP2 (100 and 50 kDa, respectively). Significantly, the enzymatic activity of the gingipains is not susceptible to inhibition by human plasma proteinase inhibitors (5). For this reason one family member (RGP) can rapidly activate proteinases involved in the complement (6–8), kallikrein/kinin (9–11), and coagulation/fibrinolytic pathways (12, 13), nearly all of which require cleavage after arginyl-X residues, whereas the other (KGP) degrades fibrinogen (13). In addition, these enzymes can also inactive many of the host plasma proteinase inhibitors, essentially dysregulating each of the cascade pathways.

Because P. gingivalis cannot extensively degrade proteins, it is now believed that this organism receives its amino acid/nitrogen requirements through the fragmentation of host proteins by proteinases produced by PMNs, including those found in crevicular fluid and, more importantly, gingival connective tissues. This apparently occurs through the release and/or activation of chemotactic factors such as C5a (6) and IL-8,2 which cause PMNs to migrate toward the bacterium. However, cleavage of chemotactic receptors from the PMN surface by RGP and KGP inhibits complete migration and phagocytosis (8), with the result being the death and disintegration of these cells and the release of their potent proteolytic and oxidative enzymes, including elastase, cathepsin G, and myeloperoxidase.

The function of PMNs is primarily associated with the eradication of bacterial infections and involves a complex interaction with cytokines, which are produced by epithelial cells, fibroblasts, macrophages, and lymphocytes. In particular, TNF-α, a proinflammatory cytokine, not only induces the production of chemotactic signals (IL-8), which result in the recruitment of PMNs to infected sites (14–16), but also is capable of acting in concert with these signals to enhance PMN function (17–20). Although the presence of TNF-α has been demonstrated in both the crevicular fluid and gingival tissues at infected sites of patients with periodontitis through enzyme-linked immunosorbent assay or in situ hybridization methodology (21–25), these procedures are incapable of measuring functional protein. Thus, the question arises as to whether TNF-α post-secretion is biologically active.

Here, we report that members of the gingipain family are capable of degrading TNF-α. Additionally, we identify the TNF-α, tumor necrosis factor-α; HRGP, adhesion domain containing Arg-gingipain 1; RGP2, Arg-gingipain 2; KGP, Lys-gingipain; HNE, human neutrophil elastase; cat G, cathepsin G; pNA, p-nitroanilide; Suc, succinyl; PVDF, polyvinylidene difluoride; IL, interleukin; Tricine, N-tris(hydroxymethyl)methylglycine.

* This work was supported in part by Grant DE-09761 from the NIDR, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¶ The abbreviations used are: PMN, polymorphonuclear leukocytes; KGP, Lys-gingipain; HNE, human neutrophil elastase; cat G, cathepsin G; pNA, p-nitroanilide; Suc, succinyl; PVDF, polyvinylidene difluoride; IL, interleukin; Tricine, N-tris(hydroxymethyl)methylglycine.

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cleavage points made by the individual gingipains within the TNF-α molecule. More importantly, these observations correlate with the loss of the TNF-α cytolytic activity. Significantly, host proteinases, human neutrophil elastase (HNE), and cathepsin G (cat G) do not degrade TNF-α and thus do not affect this specific activity. These results suggest that pathogenic bacterium may use proteolysis as a means of dysregulating cytokine activity, ultimately contributing to the evasion of host defenses.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—The mouse L929 connective tissue cell line was obtained from the American Type Culture Collection. Cells were grown at 37 °C in RPMI 1640 medium supplemented with 5% fetal bovine serum and antibiotics (Sigma). Highly purified TNF-α was a kind gift of Bayer corporation (Berkeley, CA). Three major forms of gingipain, HRGP, RGP1, and KGP, were purified according to the method of Pike et al. (26). Cathepsin G and HNE were purified as described previously (27). Polyclonal antibodies against human recombinant TNF-α were obtained from Genzyme (Cambridge, MA), and goat anti-rabbit IgG conjugated to horseradish peroxidase was purchased from Pierce. H-ο-Pro-Arg-chloromethylketone was from Bachem Biosciences (Philadelphia, PA). Nα-benzoyl-ο-Arg-pNA, methoxy-Suc-Ala-Ala-Pro-Val-pNA, and Suc-Ala-Ala-Pro-Arg-pNA were from Sigma, whereas carboxybenzoyl-Lys-pNA was purchased from NovaBiochem (La Jolla, CA).

Enzyme Activity Assay—The amidolytic activity of the purified gingipains HRGP, RGP2, and KGP was determined with either Nα-benzoyl-ο-Arg-pNA or carboxybenzoyl-Lys-pNA. Samples were preincubated in 200 mM Hepes, 5 mM CaCl2, and 10 mM cysteine, pH 7.6, for 5 min, 37 °C and then assayed for amidase activity with 1 mM substrate. The formation of p-nitroaniline was monitored spectrophotometrically at 405 nm. Amidolytic activities of the host enzymes HNE and cat G were measured spectrophotometrically using the substrates methoxy-Suc-Ala-Ala-Pro-Val-pNA for HNE and Suc-Ala-Ala-Pro-Arg-pNA for cat G.

Biological Assay for TNF-α—The cytolytic activity of TNF-α and fragments of TNF-α was monitored by the method of Mathews and Neale (28). Briefly, L929 cells were grown in 96-well flat bottom plates (Falcon Plastics) at 3 × 104 cells/ml and incubated with a serially diluted test sample of TNF-α or digested reaction mixtures of TNF-α (equivalent to a final concentration of 0.0575 ng/ml active TNF-α), a cell control, and an antibody control (a rabbit monoclonal antibody). After 24 h of incubation, actinomycin D (final concentration) was added to all wells, and cells were maintained for 16 h to ensure near complete cell lysis. The plate was washed, the remaining cells were fixed with 5% formaldehyde, and cell lysis was detected by staining the plates with a 0.2% solution of crystal violet. Stained cells were solubilized with 33% acetic acid, and the absorbance was measured spectrophotometrically as the percentage of control.

Immunoblot Analysis—TNF-α digest samples were subjected to 16% SDS-Tricine polyacrylamide gel electrophoresis (29), followed by electrophoretic transfer to nitrocellulose membranes. The primary antibody, rabbit anti-human recombinant TNF-α serum, was diluted 1:1000 prior to use. The secondary antibody, goat anti-rabbit IgG conjugated to horseradish peroxidase, was diluted 1:12,000. Immunoblots were visualized by chemiluminescence (Amersham Corp.).

Time Course Digest—Enzymatic digestion of TNF-α was performed at E:S molar ratios of 1:25 or 1:100. Briefly, reaction mixtures containing 1180 nM TNF-α and either 47.2 or 11.8 nM active enzyme in digest buffer (200 mM Hepes, 5 mM CaCl2, 10 mM cysteine, pH 7.6) were allowed to proceed for the indicated times, whereupon enzyme activity was extinguished by the addition of the cysteine proteinase inhibitor PFPNc. Each time point was divided such that 1 μl was set aside for biological assays, with the remainder being utilized for immunoblot analysis.

Amino Acid Sequence Determination—Digestion of TNF-α (2 μg) by gingipain HRGP, RGP2, or KGP was performed in digest buffer at 37 °C for 120, 10, or 120 min, respectively, at an E:S molar ratio of 1:100. Peptides from TNF-α were resolved by 16% SDS-Tricine polyacrylamide electrophoresis, electrotransferred to PVDF membrane, and stained with Coomassie Blue. Specific bands were excised and subjected to automated Edman sequence analysis using the Applied Biosystems 494 Protein Sequencer (Foster City, CA).

RESULTS

To investigate the potential immunomodulatory activities of cysteine proteinases elaborated from P. gingivalis, we first examined the ability of homogeneous preparations of three gingipains (HRGP, RGP2, and KGP) to degrade TNF-α. Fig. 1 illustrates cleavage of TNF-α by gingipains as a function of time, with protein degradation being monitored by immunoblot analysis, utilizing a polyclonal antibody raised against human recombinant TNF-α. When reaction digests at an E:S molar ratio of 1:25 were incubated at 37 °C for up to 8 h, we observed that all three gingipains were capable of degrading TNF-α. More specifically, RGP2 was capable of significantly reducing the level of the 17-kDa TNF-α band within 10 min, whereas both gingipain HRGP and KGP required additional time, with complete degradation by 240 min. As a comparison study, similar experiments were performed using homogeneous preparations of the host neutrophil serine proteinases HNE and cat G. Significantly, no detectable degradation of TNF-α was observed with either enzyme as determined by immunoblot analysis (Fig. 2).

To test whether the proteolytic degradation of TNF-α correlated with decreased biological activity of the cytokine, digest reactions were also monitored for residual biological activity in a TNF-α-specific cytolytic assay. Using aliquots from the digest mixtures above, samples were diluted to within the working range (0.080 pg/ml) of the assay (Fig. 3). Gingipain RGP2...
TNF-α Cleavage by Gingipains

Fig. 2. Lack of effect of host serine proteases cat G (A) and human neutrophil elastase (B) on TNF-α. Details are as in Fig. 1.

Fig. 3. Elimination of TNF-α bioactivity by treatment with gingipains, HRGP (A), RGP2 (B), and KGP (C). Bioactivity of TNF-α was measured in a cytolytic assay using mouse fibrosarcoma cell line L929. Cells were treated with digest reaction mixtures (described above) diluted to an equivalent final assay concentration of mature TNF-α at 37.5 pg/ml and incubated for 20 h at 37 °C. Conditioned media and cell debris were removed and discarded. Remaining cells were fixed using 33% acetic acid. Optical density was measured at 562 nm.

Reduced TNF-α activity by 75% within 10 min (Fig. 3B), whereas both HRGP and KGP (Fig. 3, A and C, respectively) were significantly more sluggish, requiring 4 h to eliminate all TNF-α activity. When parallel activity studies were performed using the host proteinases HNE and cat G, no significant loss in cytolytic activity was detected (data not shown), again correlating with the immunoblot result. Degradation and inactivation by gingipains was also confirmed in parallel experiments using decreased molar ratios of enzyme to substrate (1:100), albeit at a slower rate.

Fig. 4. Defined cleavage sites in TNF-α by gingipains. Gingipains inactivate TNF-α by hydrolysis of Arg-X or Lys-X peptide bonds (HRGP, RGP2, and KGP, respectively). Digestion mixtures consisted of an E:S molar ratio of 1:100. The TNF-α fragments were separated on gel SDS-Tricine polyacrylamide gel electrophoresis, electrotransferred to PVDF membrane, stained with Coomassie Blue, excised from the membrane, and subjected to N-terminal amino acid sequence analysis.

We next investigated the sites of proteolytic cleavage within the TNF-α molecule by the individual gingipains. The specific digestion conditions for each proteinase were optimized so as to get several cleavage products after separation by SDS-polyacrylamide gel electrophoresis, electrotransfer to PVDF membrane, and Coomassie Blue staining. Both HRGP and RGP2 were found to cleave TNF-α after Arg7 and Arg8 (Fig. 4). A third point of cleavage, determined only for RGP2, occurred at residue Arg2. Points of cleavage by KGP were at Lys63, Lys90, and Lys98.

DISCUSSION

Periodontopathic bacterial infection activates the host inflammatory response, leading to the rapid infiltration of PMNs along with an increased flow of crevicular fluid. Significantly, substances from the bacteria initiate and drive the response through the up-regulation of inflammatory mediators such as cytokines, e.g. IL-8, that are synthesized and released from resident fibroblasts, endothelial cells, and epithelial cells, in addition to leukocytes and lymphocytes. These mediators play a major role in amplification and perpetuation of inflammation, as well as in the destruction of host gingival tissues (30).

The gingipains, RGPs and KGP, are believed to be key virulence factors elaborated from P. gingivalis, the Gram-negative bacterium most often associated with adult periodontitis (31, 32). Significantly, these enzymes have been shown to activate several proteolytic cascades associated with the host inflammatory response, including those involved in the complement (6–8), kallikrein/kinin (9–11), and coagulation/fibrinolytic pathways (12, 13). Such activities, together with an ability to inactivate host plasma proteinase inhibitors, can clearly lead to a dysregulation of the inflammatory response.

The proinflammatory cytokine TNF-α is synthesized by cells in response to inflammatory mediators. In addition, it may induce the production of other cytokines, including IL-1 and IL-8 (14–16). Although up-regulated levels of TNF-α at infected periodontal sites, as compared with healthy gingival tissue, have been demonstrated through both enzyme-linked immunosorbent assay and in situ hybridization studies (21–25), the activity of this cytokine was not tested, and therefore the results are difficult to evaluate. In the present study we analyzed the ability of three individual gingipains, HRGP, RGP2, and KGP, to digest TNF-α. We chose a pH value of 7.6 for these experiments, primarily because the pH of the crevicular fluid at inflamed sites has been shown to be more alkaline (pH 7.4–8.5) than that of clinically healthy sites (pH 6.7–7.2) (33–36). Significantly, both RGPs reduced the level of the 17-kDa TNF-α band within 10 min, at an E:S ratio of 1:25. In contrast, KGP needed nearly 4 h for complete digestion of the TNF-α band.

During our studies on gingipain inactivation of TNF-α, we identified the points of cleavage associated with each enzyme. Previous studies, reviewed in Goh and Porter (37), demonstrated that the first nine residues of the TNF-α N terminus
are not necessary for biological activity, although a 3.7-fold increase in activity was observed upon deletion of the first seven residues of the N terminus. Both HRGP and RGP2 initially cleaved after residues Arg\textsuperscript{2} and Arg\textsuperscript{6}. RGP2 additionally caused fragmentation at Arg\textsuperscript{32}, a residue important for biological activity because a conserved substitution at this position results in a loss of activity (37). Although cleavage at Arg\textsuperscript{32} by HRGP was not confirmed by this method, the fragmentation pattern was similar to that of RGP2. For KGP, cleavage occurred after residues Lys\textsuperscript{63}, Lys\textsuperscript{98}, and Lys\textsuperscript{90}. Taken together these results demonstrate that the gingipain cysstein proteinases are capable of not only trimming the N terminus of TNF-\(\alpha\) but also of cleaving the polypeptide at several internal residues buried within the molecule, thus rendering the proinflammatory cytokine inactive.

It is significant that in these studies protein degradation correlated with the loss of TNF-\(\alpha\) catalytic activity (Fig. 3), which was significantly reduced within 10 min upon treatment with gingipain RGP2. Additional incubation did not reduce activity further. As expected from the protein fragmentation pattern, catalytic inactivation by either HRGP or KGP occurred at a slower rate but was still complete within 4 h. Despite the apparent disappearance of the 17-kDa substrate (Fig. 1C), these samples possessed essentially 100% biological activity (Fig. 3C), suggesting an artificially low representation of substrate and/or limitation of immunodetection method. Nevertheless, these results, taken in total, indicate that TNF-\(\alpha\) is rapidly degraded and inactivated by the gingipains tested, albeit most rapidly by RGP2.

In addition to non-host proteinases we also investigated the ability of the host PMN proteinases HNE and cat G to degrade TNF-\(\alpha\), because these enzymes are also likely to be present at inflammatory sites. Significantly, they were ineffectual in both degradation (Fig. 2) and inactivation, suggesting that TNF-\(\alpha\) is resistant to proteolytic digestion by PMN-derived enzymes. We are continuing these studies to determine whether TNF-\(\alpha\) and/or other cytokines are also refractory to proteinases secreted by host cells, in general, and more specifically to those classified as metalloproteinases because the latter are secreted by numerous cell types that would be present at inflammatory sites.

In conclusion, we have demonstrated that the bacterial cysteine proteinases elaborated by \textit{P. gingivalis} are capable of digesting TNF-\(\alpha\), an important inflammatory mediator. This strongly suggests a local dysregulation of the cytokine network and, ultimately, interruption of the host defense response, including the dysfunction of PMNs. Moreover, this study supports the belief that proteolysis can be used as a mechanism of virulence by pathogenic organisms, ultimately leading to host immune evasion.

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J. Biol. Chem. 1998, 273:6611-6614.
doi: 10.1074/jbc.273.12.6611

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