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Proteolysis of Human Monocyte CD14 by Cysteine Proteinases (Gingipains) from Porphyromonas gingivalis Leading to Lipopolysaccharide Hyporesponsiveness

Shunji Sugawara,† Eiji Nemoto,‡ Hiroyuki Tada,*,† Kensuke Miyake,‡ Takahisa Imamura,§ and Haruhiko Takada*‡

Cysteine proteinases (gingipains) elaborated from Porphyromonas gingivalis exhibit enzymatic activities against a broad range of host proteins and are considered key virulence factors in the onset and development of adult periodontitis and host defense evasion. In this study, we examined the ability of arginine-specific gingipains (high molecular mass Arg-specific gingipain (HRGP) and Arg-specific gingipain 2) and lysine-specific gingipain (KGP) to cleave monocyte CD14, the main receptor for bacterial cell surface components such as LPS. Binding of anti-CD14 mAb MY4 to human monocytes was almost completely abolished by 0.3 μM HRGP and KGP treatments for 15 min, and 1 μM RGP2 for 30 min. In contrast, the expressions of Toll-like receptor 4, and CD18, CD54, CD59, and HLA-A, -B, -C on monocytes were slightly increased and decreased, respectively, by 0.3 μM HRGP and KGP. This down-regulation resulted from direct proteolysis, because 1) gingipains eliminated MY4 binding even to fixed monocytes, and 2) CD14 fragments were detected in the extracellular medium by immunoblot analysis. Human rCD14 was degraded by all three gingipains, which confirmed that CD14 was a substrate for gingipains. TNF-α production by monocytes after HRGP and KGP treatments was decreased at 1 ng/ml, but not at 20 μg/ml LPS, indicating that gingipains inhibited a CD14-dependent cell activation. These results suggest that gingipains preferentially cleave monocyte CD14, resulting in attenuation of the cellular recognition of bacteria, and as a consequence sustain chronic inflammation. The Journal of Immunology, 2000, 165: 411–418.

A n oral chronic inflammation, i.e., periodontal disease, is one of the major diseases afflicting mankind and caused by a bacterial infection leading to gingival inflammation, destruction of periodontal tissues, loss of alveolar bone, and culminating in tooth loss (1, 2). Porphyromonas gingivalis has been implicated as a principal bacterium not only in adult periodontitis, but also in rapidly progressive periodontitis (1, 2). P. gingivalis possesses a number of putative virulence factors such as LPS, fimbriae, toxic products of metabolism, and proteinases, all of which enable this anaerobe to cause the disease either directly or indirectly by activation of host cells to release inflammatory mediators (3).

It is now clear that all of the trypsin-like proteinase activity of P. gingivalis is due to two cysteine proteinases (4). Two types of cysteine proteinases specific for Arg-X (50 and 95 kDa) (5) and Lys-X (105 kDa) bonds have been purified and characterized and are referred to as arginine-specific gingipain (RGP)3 (3) and lysine-specific gingipain (KGP) respectively (6). The 95-kDa high molecular mass RGP (HRGP or HRgpA) differs from the 50-kDa RGP (RGP2 or RgpB) in that the protein noncovalently complexes with the hemagglutinin/adhesin domain in the same manner as KGP. It has been shown that gingipains play a critical role in the onset of inflammation through enhancement of vascular permeability by activation of the kallikrein/kinin pathway (7, 8), dysregulation of plasma clot formation (9–11), activation of complement components (12), and modification of neutrophil function (13). Gingipains are also indispensable for the expression of P. gingivalis fimbriae, an important cell surface structure of this bacterium for adhesion and colonization, through normal processing of immature fimbriin (14).

CD14, mainly expressed on monocytes, functions as a major receptor for LPS (15, 16). CD14 also exists in serum as a soluble protein (soluble CD14), and a complex of soluble CD14 and LPS activates CD14-negative cells, such as endothelial and epithelial cells (17, 18). We showed recently that human gingival fibroblasts heterogeneously express CD14 and the CD14-expressed fibroblasts secrete IL-8 in response to LPS in a CD14-dependent manner (19, 20). It was shown that CD14 acts as a pattern recognition receptor for many bacterial components in addition to LPS (21, 22). As CD14 is a 55-kDa GPI-anchored membrane protein that lacks transmembrane and cytoplasmic domains (23), CD14 itself does not elicit intracellular signaling (16). The Toll-like receptors (TLRs) of vertebrates, homologues of Drosophila Toll, have been implicated in the innate immune response in vertebrates (24), and it has recently been shown that TLR2 and TLR4 mediated the entry of LPS signal into the cells of human and mouse (25–30).
Activation of monocytes through CD14/TLR by LPS and other bacterial components leads to the production of numerous inflammatory mediators, and the activated monocytes/macrophages and the released mediators function as the first line of defense against infectious bacteria. Adult periodontitis is characterized by the phases of chronic and sometimes active destruction of periodontal tissues with marked inflammation, followed by periods of remission, which leads to the hypothesis that Porphyromonas gingivalis could evoke immune surveillance by monocytes by cleaving bacterial recognition molecule(s) on monocytes using the bacterial proteinases and survive in periodontal tissues. In this study, we examined this hypothesis and showed that Porphyromonas gingivalis cytoeine proteinases, both KGP and RGP, preferentially cleave human monocyte CD14, but not TLR4, and as a consequence, inhibit a CD14-dependent monocyte activation pathway triggered by LPS.

Materials and Methods

Reagents and mAbs

Phe-Pro-Arg-chloromethyl ketone (FPR-cmk) and benzoyloxyacyononyl-Phe-Lys-chloromethyl ketone (Z-FK-cmk) were obtained from Bachem Bioscience (King of Prussia, PA), Phosphatidylinositol phospholipase C (PI-PLC) from Bacillus cereus and BSA fraction V were obtained from Boehringer Mannheim (Mannheim, Germany). Human leukocyte elastase (HLE) was obtained from Calbiochem-Novabiochem (San Diego, CA). Anti-TLR4 mAb HTA125 was raised by immunizing BALB/c mice with Porphyromonas gingivalis HG66 culture supernatant, as described by Pike et al. (6). The amount of active enzyme in each purified fraction of CD14 by gingipains

Time course of digestion

For the enzymatic digestion of rCD14, a reaction mixture containing 364 nM rCD14 and 3.6 nM active enzymes at an enzyme:substrate (E:S) molar ratio of 1:100 in 0.1 M Tris-HCl, pH 7.6, containing 150 mM NaCl and 5 mM CaCl2, was incubated at 37°C. At the time point indicated, aliquots (2.5 μl) were removed and mixed with Laemmli sample buffer. Samples were then subjected to 12.5% SDS-PAGE under reducing conditions and transferred to a PVDF membrane. The membrane was probed with polyclonal anti-CD14 Ab.

TNF-α production

PBMCs were treated with 0.3 μM gingipains at 1 × 106 cells/100 μl in the presence or absence of 3 μM specific inhibitors, i.e., Z-FK-cmk for KGP and FPR-cmk for HRGP, for 15 min at 37°C. After the treatment, cells were washed twice with RPMI 1640 medium, and seeded in a 96-well flat-bottom plate (Falcon) at 5 × 104 cells/well in the medium with 10% FCS. After 1-h incubation at 37°C, nonadherent cells were removed by washing twice with warmed medium, and adherent monocytes were stimulated with LPS for 3 h at 37°C. For the inhibition experiment with anti-TLR4 mAb HTA125, adherent monocytes were preincubated with HTA125 (10 μg/ml) or isotype control mouse IgG2a (10 μg/ml) for 30 min at 37°C and then stimulated with LPS for 3 h at 37°C. The TNF-α concentration in the supernatant was determined with an OptEIA human TNF-α ELISA set (PharMingen), according to the manufacturer’s protocol. Each sample was assayed in triplicate.

Statistical analysis

All of the experiments in this study were conducted at least three times. The data shown are representative results. Experimental values are given as means ± SD. The statistical significance of differences between two means was evaluated by Student’s unpaired t test, and p values less than 0.05 were considered significant.
Results

Preferential decrease in the surface expression of CD14 on human monocytes following P. gingivalis proteinase treatment

The effect of P. gingivalis cysteine proteinases, KGP, HRGP, and RGP2, on the expression of CD14 on human monocytes was evaluated by flow-cytometric gating forward and side scatter characteristics. A, Representative FACS profiles of CD14 expression on monocytes after 1 and 0.3 μM KGP and HRGP treatments for 15 min are shown. Isotype-matched IgG2b (C) was used as a control. B. The percent bindings of MY4 after treatments with gingipains were evaluated on the basis of the values obtained with untreated cells. The mean value of mean fluorescence intensity was 527 ± 67 for MY4 and 3.1 ± 0.2 for the isotype control IgG. Results are representative of three distinct experiments on different donors. Error bars indicate SD.

Table 1. Effect of gingipain inhibitors on the elimination of MY4 binding to monocytes induced by gingipains

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Concentration (μM)</th>
<th>KGP</th>
<th>HRGP</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPR-cmk</td>
<td>3</td>
<td>104.4 ± 6.2</td>
<td>95.8 ± 3.3**</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>68.7 ± 5.4***</td>
<td>8.4 ± 3.3***</td>
</tr>
<tr>
<td>Z-FK-cmk</td>
<td>3</td>
<td>104.8 ± 6.2</td>
<td>4.8 ± 1.7***</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>101.1 ± 3.2</td>
<td>3.3 ± 0.8***</td>
</tr>
</tbody>
</table>

* KGP and HRGP were used at 0.3 μM for 15 min. Results are the mean ± SD. **p < 0.01 compared with untreated cells. Results are representative of three distinct experiments.
The treatment with KGP and HRGP, CD14 was gradually reexpressed on the cell surface, and more than 90% of the cell surface CD14 was recovered at 48 h (Fig. 4).

**Effect of fixation on gingipain-induced CD14 elimination from the surface of human monocytes**

These results suggested that the preferential reduction of CD14 most likely resulted from direct proteolysis, but raised two other possible mechanisms of this effect. A possible mechanism of the effect of gingipains is that CD14 is shed by an endogenous enzymatic activity after monocyte activation by gingipains, as suggested by Bazil and Strominger (35). To examine this possibility, the effect of fixation with 1% paraformaldehyde on the elimination of MY4 binding to monocytes was analyzed by flow cytometry. When cells were incubated with 20 nM PMA for 60 min, a marked reduction in the binding was observed, and fixation of the cells inhibited the down-modulation of the surface CD14 (Fig. 5). These results indicate that fixation blocked the shedding of surface CD14 by the endogenous cellular enzymatic activity, as described (35). By contrast, all three gingipains efficiently down-modulated the binding of MY4 to fixed as well as unfixed monocytes, indicating that gingipains directly cleave surface CD14 on the cells.

**Immunoblot analysis of CD14 expressed and released by human monocytes**

Another possible explanation for the effect of gingipains is an augmentation of CD14 internalization. To examine this possibility, purified human monocytes were treated with KGP and HRGP, and cells and the supernatants were analyzed by Western blotting using anti-CD14 polyclonal Ab. PI-PLC, an enzyme that specifically cleaves GPI-anchored protein (23), was used as a positive control. Untreated monocytes expressed a strong 55-kDa CD14 band, whereas the band was not detected after 0.3 μM KGP and HRGP treatments and a strong reduction of cell-associated CD14 was observed after 1 U/ml PI-PLC treatment (Fig. 6A). In addition, five bands with an \( M_r \) of about 50, 40, 26, 19, and 18 kDa and one band with an \( M_r \) of about 19 kDa were revealed after 0.3 μM KGP and HRGP treatments for 15 min, respectively. In the extracellular medium, a strong 55-kDa band was detected after PI-PLC treatment, whereas a strong 48-kDa and a weak 37-kDa band, and a strong 44-kDa and a weak 19-kDa band were detected after 0.3 μM KGP and HRGP treatments for 15 min, respectively (Fig. 6B). These results indicate that gingipains directly cleave CD14 on monocytes and release CD14 fragments, and the digested fragments of CD14 still exist on the cell surface after gingipain treatments, and that the binding of MY4 to fixed monocytes was eliminated by gingipains, as shown in Fig. 3, rules out the possibility of CD14 internalization.

**Degradation of soluble rCD14 by gingipains**

To confirm that gingipains can degrade CD14, human rCD14 was incubated with gingipains at an E:S molar ratio of 1:100 at 37°C.
for up to 120 min. Fig. 7 shows that all three gingipains were capable of degrading rCD14 within 5 min, and the intensity of the rCD14 band was reduced in a time-dependent manner. The rCD14 band disappeared at 30 min on KGP and HRGP treatment, but a faint band remained until 120 min on RGP2 treatment. No fragments of rCD14, however, were detected. These results indicate that soluble rCD14 functions as a substrate of gingipains and that gingipains digest rCD14 to small fragments.

Inhibition of LPS-induced TNF-α production from human monocytes by gingipains

The observation that gingipains cleave monocyte CD14 raises the question of whether gingipains inhibit the LPS-induced production of TNF-α by monocytes, one of the major proinflammatory cytokines produced by the cells. Preincubation of human monocytes with 0.3 μM HRGP and KGP for 15 min substantially suppressed TNF-α production triggered by 1 ng/ml LPS (Fig. 8A). Pretreatment of 0.3 μM KGP and HRGP with 3 μM Z-FK-cmk and FPR-cmk, respectively, restored TNF-α production, suggesting that the proteolytic activity of gingipains was required for the inhibitory activity. By contrast, when KGP- and HRGP-pretreated human monocytes were stimulated with a higher concentration of LPS (20 μg/ml), TNF-α production was unchanged (Fig. 8B), but TNF-α production was significantly inhibited by HTA125 (10 μg/ml) (Fig. 8C). Thus, these results indicate that cleavage of CD14 on

**FIGURE 6.** Immunoblot analysis of CD14 expressed and released by human monocytes after gingipain treatments. Purified monocytes were untreated or treated with 0.3 μM KGP, 0.3 μM HRGP, or 1 U/ml PI-PLC for 15 min at 37°C. Reactions were stopped with 3 μM FPR-cmk. After centrifugation at 4°C, the medium was removed and dried by a vacuum centrifuge. Cell pellets (A) and dried medium (B) were then solubilized with Laemmli sample buffer, subjected to 10% SDS-PAGE, and transferred to PVDF membrane. The blot was probed with an anti-CD14 polyclonal Ab. Shown is one experiment representative of three.

**FIGURE 7.** Degradation of soluble rCD14 by gingipains. A reaction mixture containing 364 nM human rCD14 and 3.6 nM KGP (A), HRGP (B), or RGP2 (C) at an E:S molar ratio of 1:100 in 0.1 M Tris-HCl, pH 7.6, containing 150 mM NaCl and 5 mM CaCl₂, was incubated at 37°C. At the time point indicated, aliquots (2.5 μl) were removed and mixed with Laemmli sample buffer. Samples were then subjected to 12.5% SDS-PAGE under reducing conditions and transferred to a PVDF membrane. The membrane was probed with polyclonal anti-CD14 Ab.

**FIGURE 8.** Inhibition of LPS-induced TNF-α production from human monocytes by gingipains. PBMCs were treated with 0.3 μM KGP and HRGP in the presence or absence of 3 μM Z-FK-cmk or FPR-cmk for 15 min at 37°C, washed twice with medium, and seeded in a 96-well plate at 5 × 10⁵ cells/well. After 1-h incubation at 37°C, nonadherent cells were removed, and adherent monocytes were stimulated with 1 ng/ml LPS (A) or 20 μg/ml LPS (B) or stimulated with 20 μg/ml LPS in the presence or absence of HTA125 (10 μg/ml) or isotype control mouse IgG2a (10 μg/ml) (C) for 3 h at 37°C. The amounts of TNF-α in the supernatants were analyzed by ELISA. Error bars indicate SD. **, p < 0.01 compared with stimulant alone.
monocytes resulted in the specific suppression of the cell activation elicited by a low concentration of LPS, and that TLR4 is still functionally active after gingipain treatment.

Discussion
In the present study, we provide the evidence that P. gingivalis cysteine proteinases, gingipains, cleave human monocyte CD14, and as a consequence down-regulate LPS-induced TNF-α production by the cells. The failure of MY4 to bind human monocytes resulted from direct proteolysis of CD14, but could not be attributed to shedding or internalization of CD14, because gingipains cleaved the CD14 on 1% paraformaldehyde-fixed monocytes, and fragments of CD14 on monocytes and in the extracellular medium were detected by Western blot analysis using anti-CD14 polyclonal Ab. Human rCD14 was degraded by all three gingipains, which confirmed that CD14 was a substrate for gingipains.

Recently, Le-Barillier et al. (34) reported that HLE also cleaves CD14 on human monocytes. They showed that 30-min treatment of human monocytes with 3 μM HLE at 37°C was required for 95% reduction of MY4 binding to the cells. The present study showed that 15-min exposure of the cells to 0.3 μM KGP and HRGP almost completely eliminated the binding, indicating that bacterial proteinases, gingipains, cleave CD14 on monocytes at least 20 times more efficiently than a host proteinase, HLE. Gingipains were relatively resistant to inhibition by human serum as compared with HLE (Fig. 4). HLE was rapidly inactivated by α2-proteinase inhibitor in the serum (36). By contrast, gingipains were inhibited only by α2-macroglobulin in the serum and at a slow rate, and KGP is still 50% active on exposure to α2-macroglobulin (37), suggesting that gingipains function to cleave CD14 on monocytes more efficiently than HLE in vivo. The concentration of gingipains in the gingival crevicular fluid at inflamed sites of adult periodontitis patients was about 0.1 μM, which was the lowest concentration used in this study, and it was 10 times more than that from normal sites of these patients using the same gingipain preparations as used in this study as a standard control (K. Matsushita, T. Imamura, and I. Maruyama, unpublished data). In addition to the secreted soluble gingipains, gingipains are also either bound to the bacterial cell surface or released as outer membrane blebs (13, 38).

Therefore, it is conceivable that the local concentration of gingipains around P. gingivalis was much higher than that in the crevicular fluid, and that down-regulation of CD14 on monocytes caused by gingipains is likely to occur in vivo.

There are 21 Arg-X and 9 Lys-X bonds in the CD14 amino acid sequence (23), which are possible sites of cleavage by RGP and KGP, respectively, and it is suggested that the 65 N-terminal amino acids of CD14 are critical for the binding of LPS to CD14 and subsequent signal transduction (39, 40) and that deletion of amino acids 9–12 and 35–39 within this region eliminates MY4 binding (39). The present study showed that gingipains cleave CD14, resulting in the elimination of MY4 binding to human monocytes and reducing the release of TNF-α from the cells triggered by a low concentration of LPS (Figs. 1 and 8), indicating that gingipains efficiently eliminate at least the N-terminal regions of CD14 that are important for MY4 and LPS binding. There were more bands after KGP than after HRGP treatment for 15 min (Fig. 6A), which may be due to the number of cleavage sites, i.e., the number of Arg-X and Lys-X bonds in CD14. These observations also indicate that the elimination of MY4 binding to monocytes by gingipains, as analyzed by flow cytometry, closely correlated to the dysregulation of LPS responsiveness of monocytes, even though fragments of CD14 were still expressed on monocytes. Actually, Arg-X and Lys-X bonds existed within the 65 N-terminal sequence and around it in addition to other regions of CD14 (23). This possibility concurs with the evidence that MY4 is able to inhibit activation of monocytes initiated only by a low concentration of LPS (34). In the extracellular medium, relatively high molecular bands (48 and 37 kDa by KGP and 44 kDa by HRGP) were detected (Fig. 6B), which indicates that gingipains may cleave long CD14 fragments in the medium after 15-min treatment. By contrast, all three gingipains degraded soluble human rCD14 within 5 min, and fragments of CD14 were not detectable at any time point during digestion (Fig. 7). These observations also suggest that gingipains efficiently digest CD14 to small fragments that are not detected by Western blotting using anti-CD14 polyclonal Ab, but we do not exclude the possibility that cleavage sites of membrane-anchored CD14 may be distinct from those of soluble rCD14. Taking into account these observations, in the ability to cleave CD14, the three gingipains should rank HRGP > KGP > RGP2. HRGP and KGP differ from RGP2 in that the two proteinases have complexed with hemagglutinin/adhesin domains. This domain can bind to fibrinogen, fibronectin, and laminin (41), and reacted with phospholipids (11). Therefore, this domain may have an additional function, increasing the efficiency of the enzymatic activity by helping to adhere to the cells or CD14 itself.

As gingipains proteolytically inactivate leukocyte C5aR (12), it is possible that gingipains cleave other cell surface molecules not only on monocytes, but on other cells, resulting in dysregulation of the cells. The present study showed that expressions of CD18, CD54, CD59, and MHC class I on monocytes were reduced 20 to 30% after gingipain treatments (Fig. 2). CD59, another GPI-anchored glycoprotein expressed on a wide range of cell types including monocytes (42, 43), was not eliminated as efficiently as CD14 by gingipains, which excluded the possibility that gingipains preferentially cleave GPI-anchored molecules on the cell surface. When cells were incubated for longer period and with higher concentration (1 μM KGP and HRGP for 30 min), the expression of TLR4 was unchanged, and CD18, CD59, and HLA-A, -B, -C expressions were reduced by about 10% compared with 0.3 μM KGP and HRGP, whereas CD54 (ICAM-1) was relatively sensitive, but about 50% still remains on the cell surface after the treatment (data not shown). These results indicate that gingipains had a preference for cleaving CD14 over other polypeptides on human monocytes, although the underlying reason was unclear. The expression of TLR4 on monocytes was slightly increased by 0.3 μM KGP and HRGP treatments for 15 min, but unchanged by the gingipains after 1% paraformaldehyde fixation (data not shown), suggesting that gingipains themselves may have a function to activate monocytes, resulting in up-regulation of TLR4 expression. Experiments are underway to clarify this point.

CD14 as a GPI-anchored protein was not thought to participate directly in signaling, and TLR4 has been shown to be an LPS signaling molecule of human as well as murine cells (27–30, 44, 45). It is well known that LPS signals enter into the cells by a CD14-dependent mechanism, but at very high, supraphysiological LPS concentrations, LPS signaling occurs by CD14-independent mechanisms (16). Pretreatment of human monocytes with KGP and HRGP markedly suppressed TNF-α production triggered by a low concentration of LPS, i.e., 1 ng/ml, but not by a high concentration of LPS, i.e., 20 μg/ml (Fig. 8, A and B), and anti-TLR4 mAb HTA125 significantly inhibited TNF-α production from gingipain-treated monocytes elicited by 20 μg/ml LPS (Fig. 8C). TNF-α production from untreated monocytes triggered by a low concentration of LPS was also significantly inhibited by HTA125 as well as by MY4 (data not shown). These results suggest that 1) TLR4 is still functionally active after gingipain treatments, 2)
TLR4 transduces LPS signal in concert with CD14 at a low concentration of LPS and TLR4 can signal independently of CD14 at a high concentration of LPS, and 3) proteolysis of CD14 but not TLR4 by gingipains resulted in the specific suppression of a CD14-dependent cell activation pathway.

Recent studies have shown that gingipains can cleave proinflammatory cytokines such as TNF-α (46) and IL-8 (38) in addition to having a role in the onset of inflammation, as described above, and are implicated in immune evasion, disrupting the host cytokine network. Stimulation of gingipain-treated human monocytes with LPS after removal of residual gingipains by washing (Fig. 8) excluded the possibility that the reduced TNF-α production was due to cleavage of TNF-α itself by gingipains. CD14 is shown to be a pattern recognition receptor for many bacterial components in addition to LPS (21, 22), and monocytes can phagocytose Gram-negative bacteria by a CD14-dependent mechanism (47, 48). Therefore, the present study further supports the above-mentioned immune evasion mechanisms involving the cleaving of cell surface CD14 on human monocytes. CD14 is strongly detectable on the surface of monocytes and macrophages (49, 50) and weakly detectable on neutrophils (51) and human gingival fibroblasts (19, 20), all of which exist in periodontal tissue with periodontitis, indicating that these cell types are possibly subjected to this regulation by gingipains. It is also possible that P. gingivalis and other infective organisms may elaborate proteinases that have similar functions to gingipains. In fact, a new cysteine proteinase that cleaves kinogens and fibrinogen. J. Biol. Chem. 268:7935.

Acknowledgments

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References


