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Modulation of interleukin-8 activity by gingipains from *Porphyromonas gingivalis*: implications for pathogenicity of periodontal disease

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Abstract Gingipains are the major cysteine proteinases synthesized by *Porphyromonas gingivalis* which, in soluble form, are able to initially convert IL-8 (77 amino acid residues) to a more potent species truncated at the amino terminus, followed by slow degradation and destruction of chemokine biological activity. In contrast, the same enzymes when associated with bacterial outer-membrane blebs (vesicles), instantly degrade this chemokine. This division of enhancing and inactivating activity between soluble and membrane-bound gingipains can cause the compartmentalization of pro- and anti-inflammatory reactions to distal and proximal positions from bacterial plaque, respectively, which may explain why, despite the massive neutrophil accumulation at periodontitis sites, there is no elimination of infection.

Key words: Proteinase; Interleukin-8; Neutrophil activation; Chemokine; Periodontitis

1. Introduction

Interleukin-8 (IL-8) is a pro-inflammatory polypeptide belonging to the C-X-C family of chemokines [1,2] and is one of the most potent activators of neutrophils. This cytokine is produced by a variety of cell types including lymphocytes, monocytes, endothelial cells, fibroblasts, keratinocytes, epithelial cells and neutrophils. In addition to chemotactic activity, IL-8 has been shown to trigger both neutrophil degranulation and the respiratory burst, enhance phagocytosis, and cause the expression of CR1 and CR3 receptors on neutrophils [3–7]. While IL-8 derived from monocytes and T lymphocytes is predominantly a polypeptide of 72 amino acid residues (IL-8-72), non-immunological cells secrete a 77-residues form (IL-8-77) which is extended at the amino terminus. This form of IL-8 has significantly lower biological activity which can be enhanced by removal of the first 5–7 residues [2,4,8,9].

It is generally accepted that IL-8 is one of the major chemotactic factors responsible for neutrophil migration towards inflammatory foci [10]. For this reason it may also play a role in the influx of neutrophils into periodontal lesions during outbreaks of periodontitis, an inflammatory disease initiated by specific bacterial species residing in the subgingival plaque [11,12]. A common histological hallmark of periodontitis is a massive accumulation of neutrophils in the gingival connective tissue, the junctional epithelium and the periodontal pocket at the site of infection [13,14]. While factors responsible for this state are not fully understood, recent studies have shown that IL-8 expression in periodontal tissue can either be directly stimulated by LPS isolated from periodontopathogens or by other proinflammatory cytokines, including TNF-α and IL-1β, generated locally in response to this bacterial cell wall component [15–17].

In addition to the LPS-induced pathways of cytokine generation, pathogenic bacteria have developed various mechanisms which can alter cytokine activity at the infection site, and it is now clearly recognized that proteinases released by pathogens do significantly affect cytokine signaling networks [18]. This applies particularly to the major periodontopathogen, *Porphyromonas gingivalis*, an anaerobic bacterium which is known to produce large quantities of proteolytic enzymes with activity against a broad range of host proteins, including cytokines [18,19]. Among the numerous proteinases released by this organism, cysteine proteinases with Arg-Xaa or Lys-Xaa specificity, referred to as gingipain R and gingipain K, respectively, are recognized as important virulence factors because they appear to contribute significantly to the pathogenesis of periodontitis [20,21]. In this report we have studied the effect of these gingipains on IL-8 biological activity, because this cytokine is likely to play an important role in neutrophil accumulation at periodontitis sites.

2. Materials and methods

2.1. Reagents

Percoll, cytochalasin B, and *N*-methoxysuccinyl-Ala-Ala-Pro-Val-pNA were obtained from Sigma, while leupeptin, E-64, and DIC (3,4-dichloroisocoumarin) were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN, USA). ZFKck (benzyloxycarbonyl-Phe-Lys-CH_2OCP-2,4,6-Me3-Ph) and FPRck (Phe-Pro-Arg-chloromethylketone) were obtained from Bachem Biosciences (Philadelphia, PA, USA). The 50-kDa gingipain R (RgpB), 95-kDa gingipain R (RgpA), and gingipain K (Kgp) were purified from *P. gingivalis* as previously described [22,23], as were vesicle fractions [24].

2.2. Chemokines and antibodies

Both recombinant endothelial type IL-8-77 and monocyte IL-8-72 were purchased from BioSource International. Polyclonal antibodies against human recombinant IL-8 and goat anti-rabbit IgG conjugated with horseradish peroxidase were obtained from Genzyme (Cambridge, MA, USA) and Pierce Biochemicals (Rockford, IL, USA), respectively.

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Abbreviations: PAGE, polyacrylamide gel electrophoresis; RgpA, RgpB, high and low molecular mass forms of arginine-specific cysteine proteinase of *Porphyromonas gingivalis* (gingipains R), respectively; Kgp, lysine-specific cysteine proteinase of *P. gingivalis* (gingipain K); IL-8-72, IL-8-69, N-terminally truncated forms of IL-8 with 72 and 69 amino acids, respectively

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2.3. Proteolytic degradation of IL-8 by purified gingipains

Gingipains were activated in 200 mM HEPES, 5 mM $CaCl_2$, pH 7.6, 10 mM cysteine, for 5 min at 37°C, then mixed with IL-8-77 in the same buffer at an E/S molar ratio of 1:550 (final enzyme concentration 10 nM), and incubated at 37°C. At designated time points aliquots were removed and enzyme activity terminated by addition of leupeptin (0.5 μM final concentration) or Z-FK-ck (0.5 μM final concentration) to the Rgp or Kgp digests, respectively. Part of the reaction mixture was used to assay for IL-8 biological activity, and the remainder was analyzed by Western blotting using horseradish peroxidase conjugated polyclonal antibodies against IL-8.

2.4. Proteolytic degradation of IL-8 by vesicle-associated enzymes

The IL-8-77 (5.5 μM) was mixed with native, non-treated vesicles containing Rgp's (7 nM) and Kgp (2 nM), or vesicles pretreated with specific inhibitors, in 200 mM HEPES, 5 mM $CaCl_2$, pH 7.6, with or without 10 mM cysteine, and incubated at 37°C. After 30 min the reaction was stopped by addition of FPRck to a final concentration of 100 μM. Vesicle pretreatment with either Z-FKck (10 μM), leupeptin (100 μM), ZFKck+leupeptin, or DIC (100 μM)+E64 (10 μM) was carried out for 10 min at room temperature in buffer containing 10 mM cysteine. The concentration of active gingipain K and gingipains R associated with vesicles was determined by titration with specific inhibitors, as previously described [25].

2.5. Isolation of human neutrophils

Neutrophils were isolated from the peripheral blood of normal human volunteers using Percoll density gradient centrifugation [26]. The final cell suspension contained more than 97% neutrophils, as measured by Grünwald-Giemsa staining, with more than 95% of the cells being viable by trypan blue exclusion.

2.6. Biological assay of IL-8

Isolated neutrophils were resuspended at 5×10^6 cells/ml in Hanks' balanced salt solution and preincubated with cytochalasin B (5 μg/ml) for 10 min at 37°C. Cells were then stimulated with IL-8 (50 ng/ml), or IL-8 preincubated with gingipains, for 30 min at 37°C, centrifuged, and the supernatants tested for neutrophil elastase activity using MeO-Suc-Ala-Ala-Pro-Val-pNA (625 μM final concentration) as substrate. The amount of activity released from neutrophils under IL-8 stimulation was calculated as a percentage of total elastase activity in control cell samples lysed with hexadecytrimethyl ammonium bromide for 30 min at 37°C.

2.7. Analysis of IL-8 fragments

Samples of IL-8 were subjected to 16% SDS-Tricine polyacrylamide gel electrophoresis [27], followed by electroblotting to nitrocellulose membranes. After incubation for 1 h in a solution of rabbit polyclonal anti-human IL-8 antibodies (1 μg/ml), immunoreactive bands were visualized by incubation for 1 h with goat anti-rabbit antibodies (1:5000 dilution) conjugated to horseradish peroxidase, followed by chemiluminescent exposure. For N-terminal sequence analysis proteins were transferred to PVDF membranes, stained with Coomassie Blue, and bands of interest analyzed.

2.8. Statistical analysis

All assays were performed in triplicate with at least five different neutrophil preparations. Data are expressed as mean ± S.D. For calculation of statistical significance, the Student's paired *t*-test was used, and a *P*-value less than 0.01 was considered significant.

3. Results

3.1. Effect of gingipains on IL-8 stimulated degranulation of neutrophils

The N-terminal fragment of IL-8-77 contains 3 peptide bonds which may be susceptible to proteolysis by gingipains (Fig. 1). The cleavage at the first two bonds Arg^5-Ser^6 and Lys^8-Glu^9 by arginine- (RgpA and RgpB) and lysine-specific (Kgp) gingipains, respectively, should yield truncated IL-8 with enhanced activity, while the cleavage at the Arg^{11}-Cys^{12} bond should destroy this cytokine biological activity [28]. To test this hypothesis a mixture of IL-8-77 with either RgpA, RgpB or Kgp, incubated for different time intervals, was assayed for its ability to stimulate neutrophil degranulation (Fig. 2). After only 5 min incubation all three gingipains enhanced IL-8-77 activity to a level characteristic of the 72 residues form. In the cases of RgpA and Kgp prolonged incubation resulted in a progressive decrease in cytokine activity which was totally lost after 600 min of digestion. In contrast, treatment of IL-8-77 with RgpB caused a sustained enhancement of activity for at least 60 min and even after 10 h of incubation the cytokine digest had a significant ability to stimulate neutrophil degranulation.

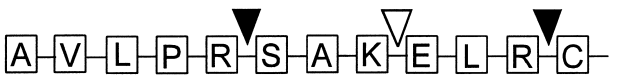

Fig. 1. N-terminal amino acid sequence of IL-8-77 with marked putative cleavage sites for gingipains R (▼) and gingipain K (▽).

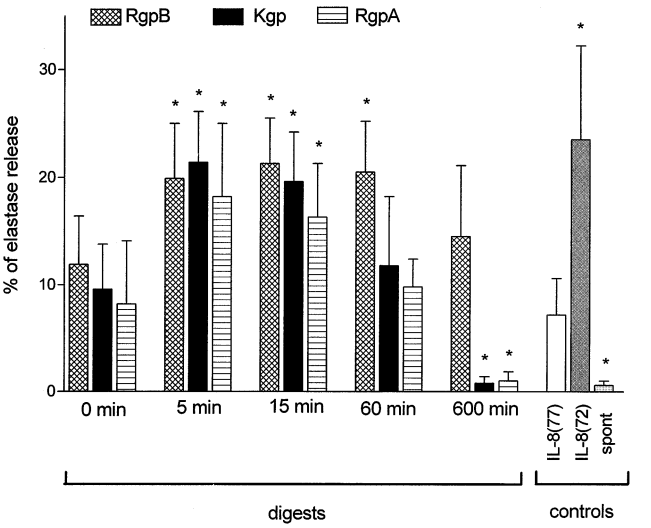

Fig. 2. Elastase release from neutrophils stimulated with gingipains digest of IL-8-77. RgpA: 95-kDa arginine-specific cysteine proteinase, a complex of a 50-kDa catalytic domain with hemagglutinin/adhesin domains; RgpB: a single chain 50-kDa proteinase; Kgp: 105-kDa lysine-specific proteinase, a complex of a 60-kDa catalytic domain with hemagglutinin/adhesin domains. The amount of activity released from neutrophils under IL-8 stimulation was calculated as a percentage of total elastase activity in control cell samples lysed with hexadecytrimethyl ammonium bromide for 30 min at 37°C. Data are presented as mean ± S.D. $^*P < 0.01$ vs. IL-8-77 control.

3.2. Proteolytic activation of IL-8-77 by individual gingipains

When recombinant IL-8-77 was digested with gingipains at a molar ratio 550:1 and the reaction mixture analyzed by immunoblotting, it was found that there was a time-dependent generation of IL-8-derived cleavage products (Fig. 3) which correlated with the observed changes in IL-8 bioactivity in the reaction mixtures. At the zero time point only a single immunoreactive band corresponding to IL-8-77 was observed, indicating that the cytokine did not undergo degradation during boiling in reduced SDS-PAGE treatment buffer. After 5 min digestion, however, an immunoreactive band with electrophoretic mobility corresponding to that of IL-8-72 appeared in samples incubated with any of the three gingipains, and this paralleled the neutrophil stimulating activity found for the truncated form of IL-8-72 (Fig. 2). Amino-terminal

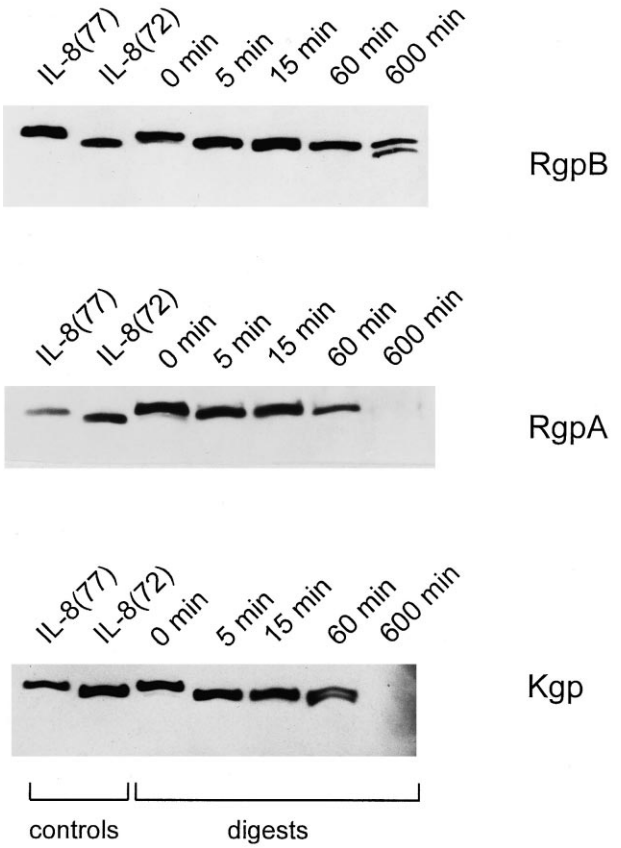

Fig. 3. Western blot analysis of IL-8-77 after incubation with gingipains.

sequence analysis of IL-8-77 cleavage products obtained after 5 or 15 min incubation with either RgpA, RgpB, or Kgp indicated that its N-terminus was processed at the Arg^5-Ser^6 and Lys^8-Glu^9 peptide bonds by Rgp's and Kgp, respectively. Incubation of IL-8-77 with Kgp for 60 min yielded two distinct immunoreactive fragments which differed in molecular mass but had the same N-terminal sequence (ELRCQCIKTY...). Similarly, two molecular mass IL-8-77 derivatives with the same N-terminal sequence (SAKELRCQICI...) were generated when the chemokine was incubated with RgpB for 600 min, both digestions indicating the degradation of the molecule within the C-terminal region. In contrast to RgpB, which contains the catalytic domain alone [23], prolonged digestion with RgpA and Kgp (complexes of individual catalytic and highly homologous hemagglutinin/adhesin domains [22]), resulted in total IL-8-77 degradation and annihilation of its neutrophil stimulating activity (Figs. 2 and 3).

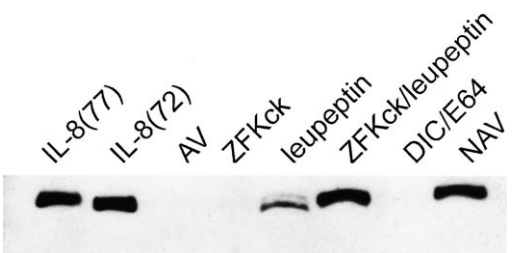

Fig. 4. Western blot analysis of IL-8-77 after incubation with *P. gingivalis* vesicles. 5.5 μM IL-8-77 was digested with vesicles containing 7 nM and 2 nM of Rgp's and Kgp, respectively. AV: Activated vesicles alone, or activated and treated with either ZFKck (10 μM), or leupeptin (100 μM), or ZFKck/leupeptin (10 μM/100 μM), or DIC/E64 (100 μM/10 μM). NAV: Non-activated vesicles.

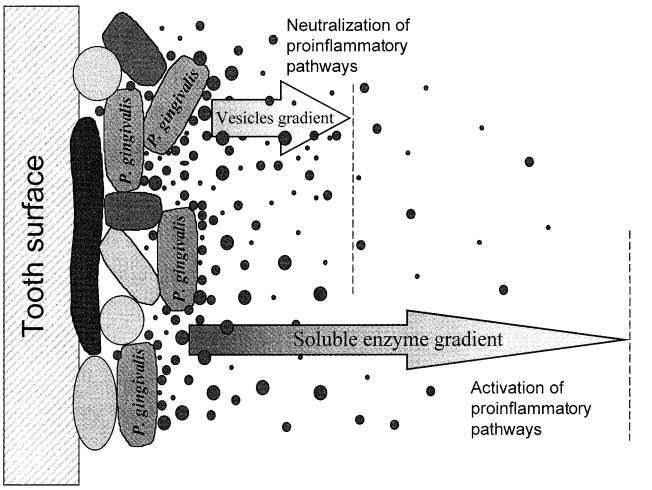

Fig. 5. Schematic representation of putative compartmentalization of pro- and anti-inflammatory reactions to distal and proximal positions from bacterial plaque induced by a different range of diffusion of soluble and vesicles (small filled circles) associated gingipains.

3.3. Processing of IL-8-77 by vesicle associated enzymes

In addition to the secretion of soluble enzymes, a significant amount of proteolytic activity elaborated by *P. gingivalis* is either bound to bacterial cell surfaces or released as outer-membrane blebs, often referred to as vesicles. Vesicles which either stay attached to the cell surface or diffuse away into the periodontal pocket drastically enlarge the bacterial surface and are considered important virulence factors [29]. In addition to gingipains [30] they carry other proteolytic enzymes [25] which may also activate and/or degrade IL-8-77. Thus, we determined the effect of vesicle-bound proteinases on the activity of this chemokine. It was clearly shown that IL-8-77 was very sensitive to degradation by vesicle-bound proteinases, and this activity was absolutely dependent on the presence of reducing agents (Fig. 4), implying a cysteine proteinase(s) as the chemokine digesting enzyme(s). This observation was further corroborated by the fact that DIC did not prevent IL-8 fragmentation, eliminating the possibility of a serine proteinase being involved in this process. Significantly, E-64 was also without effect on IL-8 degradation, ruling out the option of chemokine cleavage by periodontain, a newly described cysteine proteinase of *P. gingivalis* which is homologous to streptopain, because this enzyme activity is strongly inhibited by this compound (manuscript submitted). All of these data suggest that vesicle-bound gingipains, which are refractory to inhibition by DIC and E-64 [22,25,31] and occur at 7 nM (Rgp's) and 2 nM (Kgp) concentration in vesicles, are responsible for IL-8 cleavage. Indeed, using vesicles pretreated with leupeptin and ZFKck, inhibitors specific for Rgp's and Kgp, respectively, no degradation occurred, strongly supporting this conclusion. Moreover, data showing that IL-8 degradation could be significantly reduced by leupeptin are again in support of the hypothesis that vesicle-bound Rgp's are the major proteinases responsible for chemokine degradation.

4. Discussion

Cytokines are indispensable for the maintenance of both innate and acquired immunity. Therefore, an understanding of how bacteria can affect cytokine function at sites of infection may explain the pathological outcome of many diseases, including periodontitis [31,32]. In the cytokine network operating in the periodontium and gingiva a crucial role can be assigned to chemokines of the CXC family because they are primarily responsible for the recruitment of neutrophils. These phagocytic cells are not only involved in periodontal homeostasis [14,33,34], but also in tissue damage, especially if accumulated in excessive numbers and activated to release proteinases and oxidants [35,36]. In clinically healthy periodontal tissue a gradient of IL-8 expression is normally utilized in directing neutrophils to sites of bacterial colonization [37], and this low level inflammation is important in the maintenance of healthy tissue. This balance, however, is apparently disturbed when products released from subgingival bacterial plaque trigger fully blown inflammation, accompanied by massive influx of neutrophils, to form a leukocyte wall which becomes interposed between the plaque and junctional or pocket epithelium [14].

Despite neutrophil accumulation in periodontitis sites there is usually no elimination of infection, and destruction of connective tissue can occur. A growing body of evidence indicates that a disturbance of the cytokine network by proteolytic enzymes released by *P. gingivalis* may contribute to this state. It has already been shown that proteinases from this periodontopathogen can degrade IL-1β, IL-6 [38,39] and TNFα [40]. In this report we have presented data which suggest that *P. gingivalis* may also significantly manipulate the normal recruitment of neutrophils by chemokines through very efficient proteolytic processing and activation of IL-8-77 at the Arg^5-Ser^6 and Lys^8-Glu^9 peptide bonds by Rgp's and Kgp, respectively. In agreement with previous reports [3,9] the N-terminus truncated forms of IL-8-72 or IL-8-69 have a 2–3-fold higher neutrophil activating activity than the parent molecule. However, IL-8-77 was shown to be very susceptible to digestion by vesicle-associated gingipains, especially Rgp's, and this is an interesting observation since this chemokine is known to be resistant to both denaturation and proteolytic degradation by a broad array of host proteinases [9,41], an apparent physiological adaptation to allow it to operate at inflammatory sites rich in oxidants and proteinases.

Significantly, the degradation and inactivation of IL-8-77 depends on enzyme localization and, evidently, gingipains associated with bacterial membranes have a profoundly altered catalytic efficiency, relative to those which are secreted. This diversity of gingipain activity may have important implications relative to neutrophil accumulation at infected periodontitis sites. Soluble enzymes may be able to diffuse far away from bacterial plaque and penetrate the periodontal tissues where they can process IL-8-77 released by epithelial cells, fibroblasts and keratinocytes, thus enhancing chemokine biological activity for neutrophils as well as generate C5a, another potent chemotactic factor [42,43]. In contrast, vesicle-bound gingipains with only a limited ability to diffuse from the plaque surface may destroy the chemotactic gradient through rapid proteolytic inactivation of IL-8 and, thus, retain neutrophils at a safe distance from subgingival bacterial plaque. Taking into account that vesicle-associated proteinases can also degrade the C5a receptor on neutrophils [44], we suggest that soluble and vesicle-bound gingipains may play an important role in protecting bacteria against killing by neutrophils through the compartmentalization of pro- and anti-inflammatory reactions distal and proximal to the bacterial plaque, respectively (Fig. 5).

Recently, it has been reported that invasion of gingival epithelial cells by *P. gingivalis* totally inhibits IL-8 production by these cells in response to a variety of pro-inflammatory stimulators [45] and, more importantly, inhibits neutrophil transepithelial migration [46]. The ability of this organism to block neutrophil migration across the intact epithelial barrier and switch off IL-8 production could have a devastating effect on innate host defence in the periodontium and may play an important role in the initial stages of infection. However, such a local chemokine paralysis is apparently overcome once the subgingival plaque is fully established, because it is known that large numbers of neutrophils accumulate in periodontal pockets [13]. Significantly, *P. gingivalis* seems to participate actively in neutrophil influx, as there is a significant correlation between the occurrence of this periodontopathogen and neutrophil elastase levels in the gingival crevicular fluid at discrete periodontitis sites [47]. At this stage of the disease other bacterial defence mechanisms must operate locally to enable *P. gingivalis* to flourish in an environment saturated with neutrophils, and we suggest that this function is executed by the anti-inflammatory activity of membrane- and vesicle-associated proteinases.

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