

# The Role of Gingipains in the Pathogenesis of Periodontal Disease

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Gingipains are trypsin-like cysteine proteinases produced by *Porphyromonas gingivalis*, a major causative bacterium of adult periodontitis. HRgpA (95 kDa) and RgpB (50 kDa), products of 2 distinct but related genes, *rgpA* and *rgpB*, respectively, are specific for Arg-Xaa peptide bonds. Kgp, a product of the *kgp* gene, is specific for Lys-Xaa bonds. HRgpA and Kgp are non-covalent complexes containing separate catalytic and adhesion/hemagglutinin domains, while RgpB has only a catalytic domain with a primary structure essentially identical to that of the catalytic subunit of HRgp. HRgpA and RgpB induce vascular permeability enhancement through activation of the kallikrein/kinin pathway and activate the blood coagulation system, which, respectively, are potentially associated with gingival crevicular fluid production and progression of inflammation leading to alveolar bone loss in the periodontitis site. Kgp is the most potent fibrinogen/fibrin degrading enzyme of the 3 gingipains in human plasma and is involved in the bleeding tendency at the diseased gingiva. HRgpA activates coagulation factors and degrades fibrinogen/fibrin more efficiently than RgpB due to the adhesion/hemagglutinin domains, which have affinity for phospholipids and fibrinogen. Gingipains degrade macrophage CD14, thus inhibiting activation of the leukocytes through the lipopolysaccharide (LPS) receptor, and thereby facilitating sustained colonization of *P. gingivalis*. Gingipains play a role in bacterial housekeeping and infection, including amino acid uptake from host proteins and fimbriae maturation. Based on the important activities of gingipains in the bacterial infection and the pathogenesis of periodontitis, the bacterial proteinases can be targets for periodontal disease therapy. Immunization with RgpB, HRgpA, or a portion of HRgpA catalytic domain attenuated *P. gingivalis* induced disorders in mice. In addition, a trypsin-like proteinase inhibitor retarded *P. gingivalis* growth specifically. Gingipains are potent virulence factors of *P. gingivalis*, and are likely to be associated with the development of periodontitis. It is, therefore, suggested that gingipain inhibition by vaccination and gingipain-specific inhibitors is a useful therapy for adult periodontitis caused by *P. gingivalis* infection. *J Periodontol* 2003; 74:111-118.

## KEY WORDS

Cysteine proteinase; gingipains; periodontitis/microbiology; periodontitis/therapy; *Porphyromonas gingivalis*.

Bacterial proteinases that digest proteins to amino acids, providing nutrients for bacteria, have been thought as non-specific degradation enzymes for host structural proteins such as matrix proteins. Bacterial proteinases were not recognized as important virulence factors for years and the research on these enzymes has lagged behind in comparison to studies on lipopolysaccharides, fimbriae, etc. Recently, in vitro and animal experiments using highly purified bacterial proteinases have shown that these proteolytic enzymes caused specific activation or inactivation of bioactive proteins, thereby disturbing host systems including bio-defense mechanisms. These activities of bacterial proteinases are advantageous for the survival of bacteria but are pathogenic to the host. Thus, bacterial proteinases are associated with the pathogenesis of infectious diseases and, therefore, are potential therapeutic targets.

Periodontitis is a chronic infectious disease that starts as an inflammation of periodontal tissues and finally causes resorption of alveolar bone and subsequent loss of teeth. *Porphyromonas gingivalis* (*P. gingivalis*) is a major causative agent of adult periodontitis.<sup>1-3</sup> This bacterium, an asaccharolytic anaerobe, produces and releases a large amount of proteolytic enzymes. A trypsin-like proteinase activity shows a close relationship with *P. gingivalis* virulence.<sup>4-6</sup> During the past decade, trypsin-like proteinases were purified and characterized at the protein level and their functions have been investigated thoroughly. Accumulated knowledge on *P. gingivalis* trypsin-like proteinases,

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named gingipains, indicates their important roles in bacterial housekeeping, the infection process, and development of periodontitis. The structure and pathogenic activities of gingipains will be reviewed along with the potential availability of gingipains as targets for therapeutic agents of periodontitis.

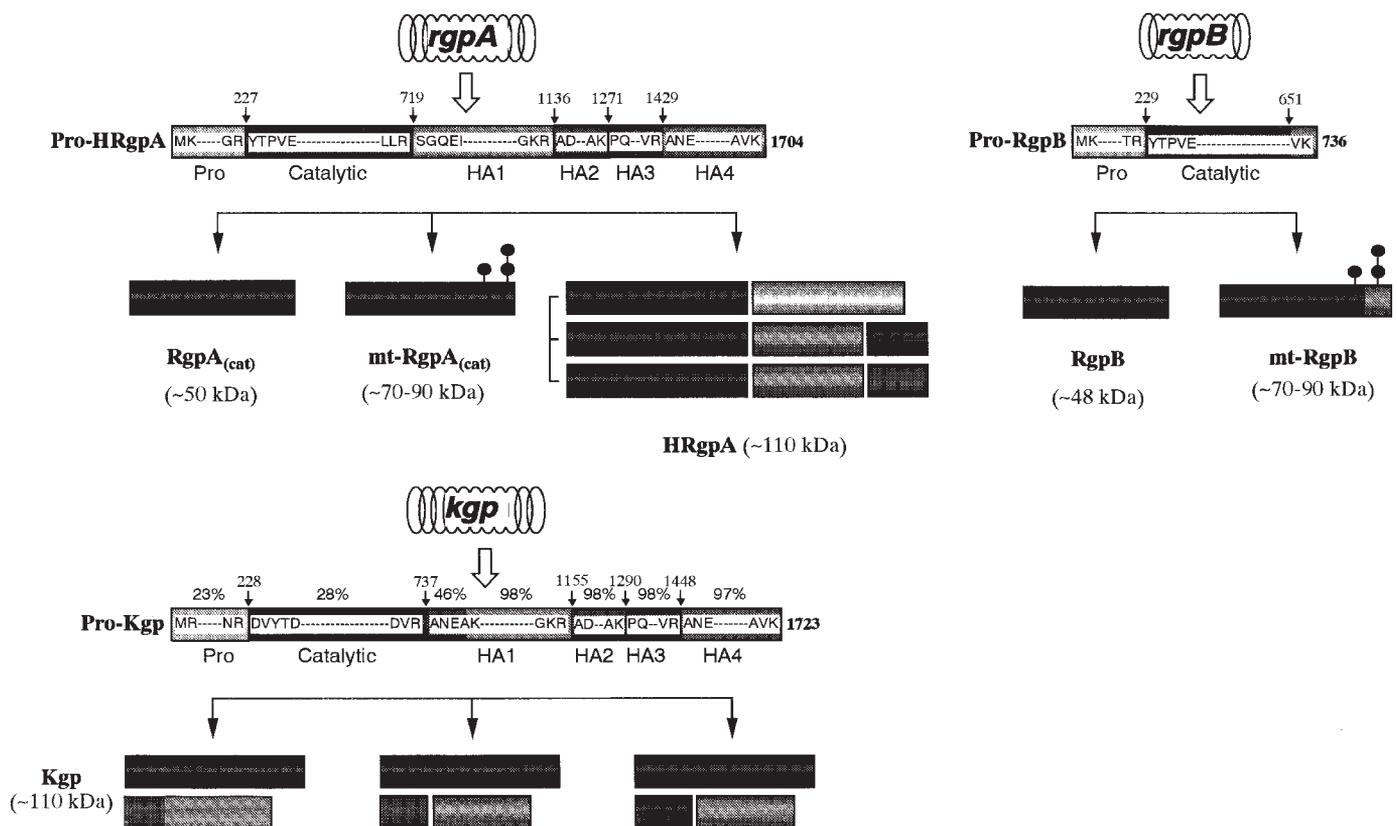
## STRUCTURE OF GINGIPAINS

The gingipains constitute a group of cysteine endopeptidases that are responsible for at least 85% of the general proteolytic activity<sup>7</sup> and 100% of the so-called “trypsin-like activity”<sup>8</sup> produced by *P. gingivalis*. Gingipains are products of 3 genes encoding cysteine proteinases and referred to as gingipain R and gingipain K, depending on a specificity for hydrolysis of either Arg-Xaa or Lys-Xaa peptide bonds, respectively.<sup>9</sup> Recently, it was suggested that the gene encoding gingipain R with hemagglutinin/adhesion domains should be referred to as *rgpA* and the gene that encodes gingipain R with-

out this carboxy-terminal domain should be referred to as *rgpB*. The name *kgp* was suggested as a reference to the gene encoding gingipain K.<sup>10</sup>

### Gingipains R Structure

The translated product of *rgpA* consists of a profragment with a signal sequence, a catalytic domain, and a hemagglutinin/adhesion domain (Fig. 1). The initial translation polyprotein is processed into at least 3 different molecular forms of the enzyme. RgpA<sub>(cat)</sub> is a form of the catalytic domain alone and is made by either aberrant proteolytic processing of the initial protein or by an interrupted transcription process.<sup>12</sup> A membrane-associated form in which the catalytic domain is modified with lipopolysaccharides is mt-RgpA<sub>(cat)</sub>.<sup>13</sup> HRgpA is the non-covalent but very stable complex of the catalytic domain and a hemagglutinin/adhesin domain(s).<sup>14</sup> In contrast, RgpB is a product of *rgpB* that is missing almost the entire section encoding the hemagglutinin/adhesin domains except for a small carboxy-terminal



### Figure 1.

Isoform construction by proteolytic processing and assembly of the translated products of *rgpA*, *rgpB*, and *kgp* genes (HG66). Open arrows denote translation of gingipain genes to their proforms. Pro, profragment; Catalytic, catalytic domain; HA1, HA2, HA3, HA3/4, HA4, hemagglutinin/adhesin subdomains. Initial and end amino acids of domains are shown. Small numbered arrows denote the cleavage site amino acid numbers and large arrows denote proteolytic processing and assembly of the mature enzyme complex, respectively. The domains with high homology are shown in the same pattern and the percentage above Pro-Kgp columns denotes homology percentage of the Pro-Kgp domains for the corresponding Pro-HRgpA domains. Dots attached to mt-RgpA<sub>(cat)</sub> and mt-RgpB denote polysaccharide chains. Molecular weights of isoforms are shown in parentheses. (Modified from Potempa et al.<sup>11</sup>)

segment, which is a single chain enzyme containing only a catalytic domain.<sup>15</sup> The translated polypeptides of the *rgpA* and *rgpB* genes share 72%, 93%, and 40% identity within the profragments, the catalytic domains, and the carboxy-terminal extensions, respectively. Attachment of (lipo)polysaccharides to RgpB apparently leads to the generation of a membrane-associated form, referred to as mt-RgpB.<sup>13</sup> However, the soluble form of RgpB purified from the culture medium of HG66 is devoid of the lipopolysaccharide component and is truncated at the carboxy-terminus in comparison with the nascent gene product (Fig. 1).<sup>16</sup> The releasing mechanism of the soluble form is still unclear, but the proteinase shedding may be due to an aberrant proteolytic cleavage of a carboxy-terminal extension containing a lipopolysaccharide attachment site.

### Gingipain K Structure

The *kgp* gene encodes a polyprotein consisting of a typical leader sequence, a profragment, a catalytic domain, and hemagglutinin/adhesin domains (Fig. 1).<sup>17</sup> The *kgp* and *rgpA* gene products from HG66 share 23% and 28% identity of the amino acid sequence within the profragment and the catalytic domains, respectively. In contrast, within the hemagglutinin/adhesin domains, the 2 gene products are highly homologous. The amino-terminal portions of HA1 of both HRgpA and Kgp share only 46% homology and, in the case of Kgp, this portion is a perfect repeat of the amino-terminal sequence of HA4 (Fig. 1). As in the case of the *rgpA* initial translation product, the nascent Kgp polyprotein is a non-covalent heteromultimeric complex of the catalytic and hemagglutinin/adhesin domains (Fig. 1).

### Structural Relationship Between Gingipains and Other Cysteine Proteinases

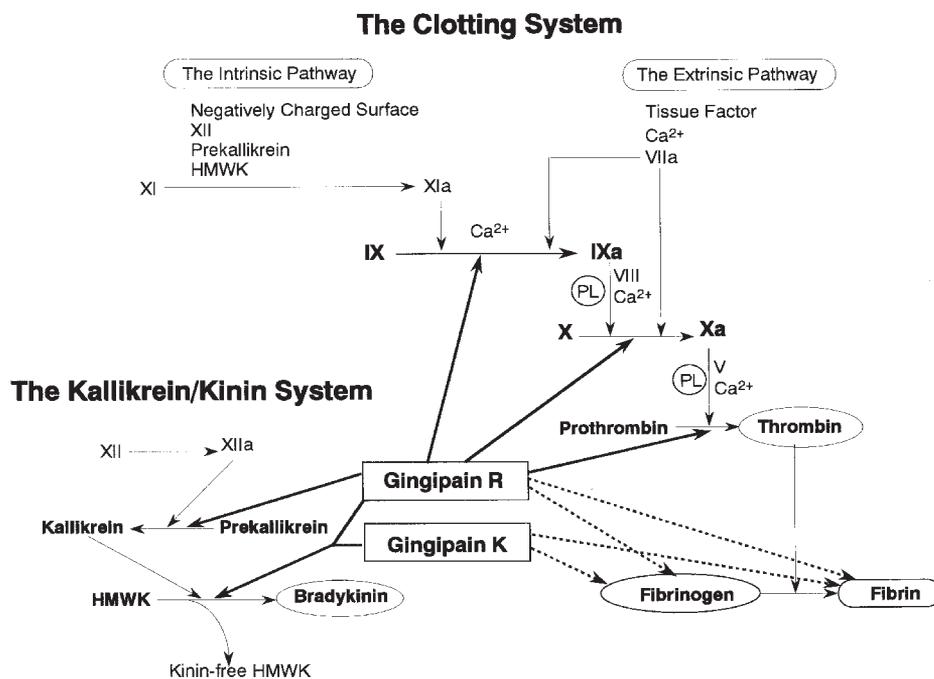
The primary structure of gingipains, in terms of their amino acid sequences, shows little significant similarity to known proteins. This indicates that gingipains are unique proteinases, and they have been assigned to a separate family (family C25) of cysteine proteinases. Despite a relatively low similarity between the amino acid sequences of the catalytic domains of gingipain R and gingipain K, the active site cysteine and histidine residues of the catalytic dyad are well conserved. Recently, it was shown that mammalian legumain (family C13), a lysosomal cysteine proteinase

specific for Asn-Xaa peptide bonds, contains a catalytic dyad in the motif, His-Gly-spacer-Ala-Cys,<sup>18</sup> which is also present in the gingipains (family C25), caspases (family C14), and clostripain (family C11). Therefore, it has been suggested that these 4 families may have similar protein folds and, accordingly, are evolutionarily related with the clan CD of cysteine proteinases.<sup>18</sup> This assumption was partially supported by the tertiary structure of crystallized RgpB. Analysis of the structure revealed that a 435-residue polypeptide chain was organized into a catalytic subdomain (the amino-terminal 340 residues) bearing topological similarities to the heterodimer of caspase-1 and an immunoglobulin-like domain (the last 95 residues).<sup>19</sup> A similar fold can be assumed for the equivalent regions of HRgpA and Kgp. This is a fascinating discovery since caspases play a central role in the process of apoptosis in animal cells.

## PATHOGENIC ACTIVITIES OF GINGIPAINS

### Activation of the Kallikrein/Kinin System

Bradykinin (BK), a nonapeptide, induces pain and vascular permeability enhancement by topical administration and causes shock by intravenous injections into experimental animals. Plasma kallikrein, converted from prekallikrein by activated Hageman factor (coagulation factor XII), releases bradykinin from high molecular weight kininogen (Fig. 2). The ability of *P. gingivalis*



**Figure 2.**

The target sites of gingipains in the kallikrein/kinin and the clotting systems. Solid arrows and dashed arrows denote activation or release, and degradation, respectively.

proteinases to activate the kallikrein/kinin pathway was first described by Hinode et al.<sup>20</sup> and Kaminishi et al.<sup>21</sup> and later was studied in detail by Imamura et al.<sup>22,23</sup> It was found that gingipains R are very potent vascular permeability enhancement (VPE) factors, inducing this activity through plasma prekallikrein activation and subsequent BK release. In this activity no significant difference was seen between HRgpA and RgpB.<sup>24</sup> In crude bacterial extracts, the VPE activity was completely abolished by the gingipain-R-specific inhibitor, leupeptin, and by anti-RgpB antibodies, indicating that gingipain R are exclusively responsible for the bacterial VPE activity.<sup>22</sup> In contrast, gingipain K was not able to induce VPE in human plasma. However, working synergistically with gingipain R, the 2 gingipains release BK directly from high molecular weight kininogen, thus mimicking the action of kallikrein (Fig. 2).<sup>23</sup> It is obvious that both gingipains are important VPE factors and potentially contribute to gingival crevicular fluid (GCF) production and edema formation at periodontitis sites infected with *P. gingivalis*. By this activity, gingipains provide a continuous supply of nutrients necessary for bacterial growth and virulence. In addition, BK may be involved in alveolar bone resorption by inducing prostaglandin production in periodontal ligament cells<sup>25</sup> and osteoblasts.<sup>26</sup> Taking into account that BK works synergistically with thrombin, interleukin-1, and an acute phase protein, haptoglobin,<sup>25,27</sup> this effect of BK should not be underestimated.

#### Activation of the Blood Clotting System

Activation of the blood coagulation system generates thrombin through the proteolytic cascade pathway. Thrombin is an extremely potent platelet activator and converts fibrinogen to a fibrin clot, thus plugging damaged vessels. Besides its central role in hemostasis, thrombin enhances vascular permeability<sup>28</sup> and induces leukocyte chemotaxis.<sup>29,30</sup> These actions potentially contribute to GCF production and leukocyte accumulation, respectively, in periodontitis sites. Thrombin stimulates prostaglandin secretion by osteoblastic cells<sup>31</sup> and potentiates lipopolysaccharide-stimulated interleukin-1 production by macrophages.<sup>32</sup> The elevated levels of both factors in GCF of adult periodontitis patients are associated with the tissue destruction process of periodontal disease.<sup>33,34</sup> Moreover, thrombin stimulates bone resorption by osteoclasts through a prostaglandin-dependent pathway.<sup>35</sup> Therefore, uncontrolled generation of thrombin is likely to be associated with the progression of periodontitis and alveolar bone resorption.

Gingipain R shortened human plasma clotting time, with HRgpA being 5-fold more efficient in comparison with RgpB.<sup>36</sup> As for the mechanism of plasma clotting promotion, these proteinases were found to activate coagulation factors IX,<sup>37</sup> X,<sup>36</sup> and prothrombin<sup>38</sup>

(Fig. 2). Consistent with the ability of plasma clotting acceleration, HRgpA was more potent than RgpB in the activation of these factors, suggesting a significant role of the hemagglutinin/adhesin domain in interaction with the factors. Notably, this function of HRgpA, not RgpB, was augmented several-fold in the presence of calcium ion and phospholipids,<sup>36-38</sup> ubiquitous cell components, and important cofactors in the clotting pathway. The phospholipid effect is the most conspicuous in HRgpA factor X activation (7- to 8-fold). Accordingly, the catalytic efficiency ( $k_{cat}/K_m$ ) of HRgpA is higher for factor X than for the other 2 factors and is comparable with that of Russell's viper venom factor X coagulant protein,<sup>36</sup> the strongest factor X activator known to date. However, the activation velocity is affected by each factor concentration, which is limited in vivo, and the prothrombin concentration in plasma is the highest (1.1  $\mu$ M). Taken into account the plasma concentrations of these zymogens, the ratio of the relative activation velocities for factor IX, factor X, and prothrombin by HRgpA was calculated from their  $K_m$  and  $k_{cat}$  values and was approximately 2: 4: 5,<sup>37</sup> while that by RgpB was 0.3: 0.1: 0.4. These data indicated that gingipain R, especially HRgpA, are potent activators of the clotting factors and primarily activate prothrombin in plasma. Considering the cascade reaction of the clotting system (Fig. 2), although the activation of upstream factor IX and factor X by the gingipains R is less than that of thrombin, it causes amplified production of thrombin, and thereby will contribute significantly to the clotting induction by gingipains R in vivo. Furthermore, gingipain R also activate protein C, leading to the consequent consumption of this clotting regulator,<sup>39</sup> thus promoting the clotting reactions triggered by the proteinases. Eventually, in periodontitis lesions it is likely that gingipain R generate a large quantity of thrombin, which is not linked to hemostasis. The multiple proinflammatory actions of thrombin will promote periodontal disease.

#### Degradation of Fibrinogen and Fibrin

Bleeding on probing, a well-known sign of periodontitis, is a widely used criterion to diagnose gingival inflammation and a commonly utilized predictor of the progression of this disease. This sign significantly correlates positively with the presence of *P. gingivalis* in the periodontal pocket,<sup>40</sup> suggesting an involvement of the bacterium-associated substance(s) in the bleeding tendency. In fact, gingipains degraded fibrinogen within minutes,<sup>41</sup> thus rendering this physiological thrombin substrate non-clottable (Fig. 2). Moreover, these enzymes solubilized fibrin gel.<sup>24</sup> Although comparable to plasmin, Kgp was less potent than gingipains R in abrogating clottability of purified fibrinogen. Nevertheless, Kgp prolonged plasma clotting time more efficiently,<sup>42</sup> indicating that in plasma Kgp inter-

acts with fibrinogen more specifically than gingipain R. Furthermore, the fact that none of the plasma proteinase inhibitors effectively suppresses Kgp<sup>43</sup> facilitates the proteinase action in plasma. The fact that the ability of *P. gingivalis* vesicles to prolong plasma clotting time was inhibited mostly by a Kgp-specific inhibitor<sup>42</sup> suggests that the fibrinogenolytic activity of the bacterium is attributed mainly to the Kgp activity. Interestingly, Kgp and HRgpA, but not RgpB, bound to fibrinogen in a specific manner,<sup>41</sup> and HRgpA destroyed this clotting protein more efficiently than RgpB,<sup>24</sup> although their catalytic domains are almost identical.<sup>14,15</sup> It is obvious that the binding of the hemagglutinin/adhesin domain to fibrinogen augments the fibrinogen cleavage by the catalytic domain. Taken together, Kgp exerts strong fibrino(geno)lytic activity in vivo which cannot be effectively controlled by host proteinase inhibitors. This action of Kgp would contribute to a bleeding tendency at periodontitis sites overwhelming the procoagulant activity of gingipain R.

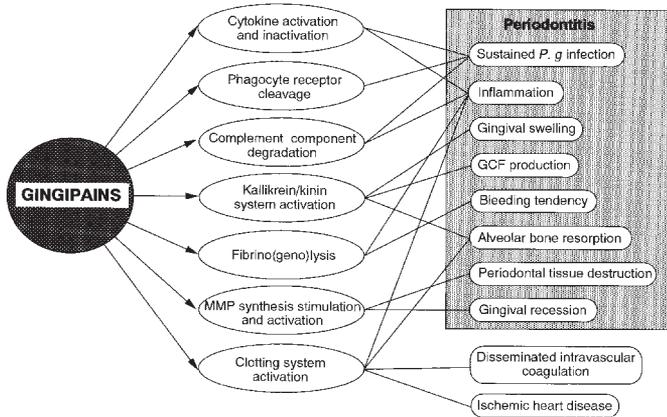
#### **Disturbance of Host Defense Systems**

Any invading pathogen is a target for the host defense system. One is constitutive and non-specific and is performed predominantly by phagocytes and activation of a part of the complement system. Another is inducible; mobilized on demand; and is composed of antibodies, activated macrophages, and cytotoxic T cells. Both bio-defense systems interact and are synchronized by the cytokine network to eliminate the invader. However, pathogenic microorganisms have acquired a variety of strategies against the attack of the host defense systems in order to survive. In the case of *P. gingivalis* the gingipains seem to play a pivotal role in the evasion of the host defense systems.

*P. gingivalis* proteinase(s), presumably gingipain R, cause complement system activation in the fluid phase through C3 convertase production by a factor D-like action,<sup>44</sup> resulting in consumption of its components. Thus, bacteriolysis through complement system activation on the surface of the bacterium would be impaired. Moreover, RgpB easily degrades C3,<sup>45</sup> the central factor of the system, thereby interfering with the production of the opsonin C3b, resulting in the insufficient generation of the phagocyte chemotactic factor C5a and the membrane attack complex. Preincubation with HRgpA reduced neutrophil chemiluminescence response by stimulation with zymogen-activated serum,<sup>46</sup> which contains the complement system activation products including C5a. Kgp and a non-tryptic serine protease associated with vesicles were found to cleave C5a receptor.<sup>47</sup> Thus, gingipains would disturb neutrophil migration to the site of *P. gingivalis* infection and protect the bacterium from phagocytosis by the leukocytes stimulated with C5a. The effect of gingipains on cell surface proteins is also seen in monocytes. Gingi-

pains degrade monocyte CD14, a major receptor for bacterial lipopolysaccharides (LPS), rendering the leukocytes hyporesponsive to LPS.<sup>48</sup> Accordingly, it is likely that the activation signal by LPS of *P. gingivalis* is insufficient for the monocytes/macrophages to act as phagocytes and secrete various cytokines which stimulate immune cells, including themselves. This may explain the defective elimination of *P. gingivalis* from the host, and consequently, the chronic inflammation at periodontitis lesions.

Interleukin-8 (IL-8), a member of C-X-C chemokine family, is a peptide of 77 amino acid residues and is produced by various cells, such as lymphocytes, macrophages, endothelial cells, and fibroblasts. Gingipain R and Kgp in soluble forms cleave this neutrophil chemotactic factor at Arg<sup>5</sup>-Ser<sup>6</sup> and Lys<sup>8</sup>-Glu<sup>9</sup>, respectively, and the products IL-8<sub>6-77</sub> and IL-8<sub>9-77</sub> are 2- to 3-fold more potent than the native IL-8. In contrast, gingipains associated with vesicles instantly degrade this chemokine, abolishing its chemotactic activity.<sup>49</sup> The proteolytic activity of gingipains is basically equal between the 2 forms, but the proteinases distribute densely on the vesicle surface, suggesting the enzyme concentration dependency of the effect. It is conceivable that at a proximal position from the bacterial plaque, where vesicle-bound gingipains are rich, IL-8 activity is primarily abrogated. However, at a distal position where vesicles are rare, free gingipains are more effective in comparison to fewer vesicle-bound enzymes, presumably augmenting IL-8 activity. In this context, massive neutrophil accumulation is induced at tissues adjacent to the plaque by truncated forms of IL-8, but the neutrophils are unable to approach the bacterium further against a gradient of decreasing IL-8 concentration. This condition may be involved in destruction of tissues around the plaque and failure of elimination of the bacterium at periodontitis sites. A similar event can occur with IL-6, an important mediator of inflammation and humoral immunity. Gingipains inactivate IL-6 rapidly by removing the carboxy-terminal peptides,<sup>50</sup> including the last 3 amino acid residues indispensable for the biological activity of this cytokine. Thus, the proteinases would produce a negative IL-6 gradient from the plaque to the adjacent tissues, which is consistent with the fact that in periodontal lesions a very low level of IL-6 was detected in the gingival tissue adjacent to the bacterial plaque, whereas significantly elevated concentrations of this cytokine are found in tissues over 6 mm away from the infected pocket.<sup>51</sup> Consequently, at the proximal sites of the plaque the inflammatory reactions mediated by IL-6 are reduced, in contrast to the distal sites, where the reactions are enhanced, leading to destruction of the periodontal tissue. In addition to IL-8 and IL-6, gingipains degrade IL-1 $\beta$ ,<sup>52</sup> tumor necrosis factor- $\alpha$ ,<sup>53</sup> and interferon- $\gamma$ .<sup>54</sup>



**Figure 3.**

The multiple pathogenic activities of gingipains and their association with periodontitis and other diseases. The lines indicate proposed links of gingipain activities to periodontitis symptoms and other diseases. (Modified from Potempa et al.<sup>11</sup>)

Gingipains could cause impaired functions of the cells associated with the bio-defense systems and dysregulation of the cytokine network. By successful execution of this strategy, *P. gingivalis* can escape from the host defense mechanisms and survive for long periods within periodontal tissue. Against this resident pathogen, the inflammatory responses continue and accumulated leukocytes sustain fruitless attack, resulting in periodontal tissue injury and progression of periodontitis. In the tissue breakdown, the participation of gingipains is also possible via induction of latent matrix metalloproteinase synthesis in fibroblasts<sup>55</sup> and activation of the zymogens.<sup>56</sup>

### Gingipains as Targets for Periodontitis Therapy

The multiple pathogenic activities of gingipains are summarized in Figure 3. The potential contribution of gingipains to the pathophysiology of periodontitis suggests availability of the enzymes as targets for the therapy of periodontal disease.

The first possibility is a vaccination therapy using gingipains for periodontitis. The immunization of primates with RgpB appeared to reduce alveolar bone loss slightly in the experimental gingivitis and ligature-induced periodontitis, but it did not suppress the emergence of *P. gingivalis* in spite of the significantly elevated level of IgG specific for both the bacterium and the gingipain.<sup>57</sup> In contrast, the immunization of mice with a peptide derived from the amino-terminal sequence of the catalytic domain of gingipains R resulted in protection from *P. gingivalis* invasion and subsequent cachexia and death.<sup>58</sup> This effect was not seen in the animals immunized with peptides derived from either the middle or the carboxy-terminal sequence of the catalytic domain or a peptide from the hemagglutinin/adhesin

domain, although the IgG titer obtained following immunization with the last peptide was comparable to that obtained following immunization with the amino-terminal peptide. This result indicates that antibodies directed to the amino terminal region of the catalytic domain of gingipains R are capable of inducing a protective immune response against *P. gingivalis* infection, suggesting a possible availability of the vaccination with the peptide for human periodontal disease.

Another possibility is the development of inhibitors specific for gingipains. Highly specific inhibitors for gingipains have not been reported yet. Recently, Matsushita et al. found that DX-9065a, a proteinase inhibitor primarily specific for activated coagulation factor X, selectively reduced *P. gingivalis* growth,<sup>59</sup> suggesting a potential therapeutic effect of gingipain inhibitors on periodontitis. Furthermore, at concentrations as low as 10  $\mu$ M, this highly safe compound inhibits both gingipain R and kallikrein generated by the proteinases in plasma, attenuating the gingipain-induced BK production. Tetracycline and its analogues, when administered to periodontitis patients, have been observed to improve their clinical parameters.<sup>60</sup> However, this effective treatment did not affect the *P. gingivalis* load at periodontitis sites.<sup>61</sup> Curiously, these antibiotics have been found to inhibit all 3 gingipains (HRgpA, RgpB, Kgp).<sup>62</sup> This result may indicate that the improvement of the clinical parameters is due to their ability to inhibit multiple gingipain actions rather than to eradicate *P. gingivalis*. Although the trial of gingipain inhibitors for periodontitis has just started, this approach may contribute to the therapy of this periodontal disease.

### ACKNOWLEDGMENT

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