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Degradation of host protease inhibitors and activation of plasminogen by proteolytic enzymes from *Porphyromonas gingivalis* and *Treponema denticola*

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Bacterial proteases may participate in the pathogenesis of periodontal diseases through their action on host proteins. In the present study, the ability of selected periodontopathogens, as well as two proteases isolated from *Porphyromonas gingivalis* and *Treponema denticola*, to degrade host protease inhibitors was evaluated. The activation of human plasminogen by the two bacterial proteases was also investigated. Proteolytic breakdown of host protease inhibitors (α-1-antitrypsin, antichymotrypsin, α_2-macroglobulin, antithrombin III, antiplasmin and cystatin C) was evaluated by SDS-PAGE. The 80 kDa trypsin-like protease of *P. gingivalis* completely digested the six protease inhibitors under investigation, whereas the 95 kDa chymotrypsin-like protease of *T. denticola* was slightly less active, more particularly on α_2-macroglobulin and cystatin C. When whole cells from a number of oral bacterial species were tested, the most significant degradation was obtained with *P. gingivalis, T. denticola, Prevotella intermedia, Prevotella nigrescens* and *Capnocytophaga* spp. *Peptostreptococcus micros* and *Propionibacterium acnes* had only some degradative activity on selected inhibitors, whereas three bacterial species, *Actinobacillus actinomycetemcomitans, Bacteroides forsythus* and *Fusobacterium nucleatum*, had no effect on the protease inhibitors. The 80 kDa protease of *P. gingivalis* demonstrated strong plasminogen activation, whereas no such activity was associated with the 95 kDa protease of *T. denticola*. This study indicates the high potential of some periodontal pathogens to destroy protease inhibitors and activate plasminogen. This may result in an uncontrolled degradation of periodontal tissues and a rapid progression of the disease.

Keywords: *Porphyromonas gingivalis*, *Treponema denticola*, bacterial proteases, plasma protease inhibitors, periodontal disease

INTRODUCTION

Periodontal diseases are probably the most common chronic inflammatory disorder in adults and often lead to tooth loss (Williams, 1990). Among over 300 bacterial species found in the oral cavity, only some, either alone or in combination, are thought to participate in the pathogenic process of periodontal diseases (Haffajee & Socransky, 1994). Although there is no doubt that bacteria represent the primary factor in the aetiology of periodontitis, the tissue destruction is also a consequence of the host response (Seymour *et al.*, 1993). The fact that a significant reduction in gingival collagen fibre density is associated with the appearance of adult periodontitis (From & Schultz-Haudt, 1963) suggests that proteases from host cells and periodontopathogens such as *Porphyromonas gingivalis* and *Treponema denticola*, may play a critical role in the disease.

Over the last ten years, increasing numbers of reports have underlined the potential importance of bacterial proteases, more particularly from *P. gingivalis* and *T. denticola*, in the pathogenesis of periodontal diseases (Grenier & Mayrand, 1993; Holt & Bramanti, 1991). Some evidence for the presence of bacterial proteases *in vivo* is also available. For example, on the basis of analyses

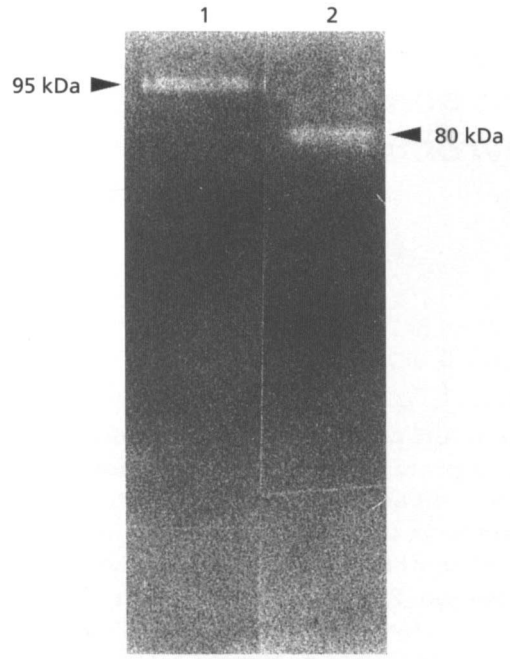

Fig. 1. Detection of proteolytic activity by zymography. Protease preparations were separated by electrophoresis on a BSA-conjugated SDS-PAGE gel. The gel was then washed, incubated and stained as described in Methods. Lanes: 1, 95 kDa protease from *T. denticola*; 2, 80 kDa protease from *P. gingivalis*.

of serum IgG responses in periodontitis patients, it has been suggested that *P. gingivalis* proteases are important antigens (Ismail *et al.*, 1988). Furthermore, a positive correlation was demonstrated between trypsin-like activity in subgingival sites and both the level of clinical disease and the numbers of *P. gingivalis* and spirochaetes (Suido *et al.*, 1988).

Previous studies have indicated that *P. gingivalis* and *T. denticola* are capable of degrading basement membrane components, such as collagen and fibronectin (Grenier & Mayrand, 1993; Grenier *et al.*, 1990; Holt & Bramanti, 1991), suggesting a direct role for these bacteria in degradation and invasion of periodontal tissues. Bacterial proteases may also participate indirectly in tissue destruction. It is well known that plasma protease inhibitors may contribute to host defences by inhibiting bacteria- and host-derived proteolytic activities. In the gingival crevicular fluid, their presence may help preserve the integrity of the periodontal tissues. The degradation of α-1-antitrypsin and α_2-macroglobulin by cells of *P. gingivalis* and *T. denticola* has been previously demonstrated (Carlsson *et al.*, 1984; Fishburn *et al.*, 1991; Herrmann *et al.*, 1985; Uitto *et al.*, 1988). C1-inhibitor, α_2-antiplasmin and antithrombin, which are involved in the control of the plasma proteinase cascade system, are also degraded by *P. gingivalis* (Nilsson *et al.*, 1985). It is suggested that inactivation of these inhibitors may enhance inflammatory responses, vascular permeability and fibrinolysis, thus contributing to the pathogenic process of periodontal disease. Plasmin activity was previously reported to increase in gingival fluid from affected periodontal sites (Hidaka *et al.*, 1981). The ability of proteases from bacteria to activate human plasminogen into plasmin may represent an additional mechanism by which these bacterial enzymes indirectly participate in tissue destruction.

In the present study, the ability of selected periodontopathogens, as well as two proteases isolated from *P. gingivalis* and *T. denticola*, to degrade host protease inhibitors was evaluated. The activation of human plasminogen by the two bacterial proteases was also investigated.

METHODS

Bacteria and growth conditions. The reference and clinical strains of oral bacterial species under investigation were: *Actinobacillus actinomycetemcomitans* ATCC 29522 and Y4, *Bacteroides forsythus* ATCC 43037 and 4067M27, *Capnocytophaga* spp. VA and III.21, *Fusobacterium nucleatum* 102.3 and CM33MB-5, *Peptostreptococcus micros* NY370 and 89A, *P. gingivalis* ATCC 33277 and W50, *Prevotella intermedia* ATCC 25611 and NY365, *Prevotella nigrescens* NCTC 9336 and SPRO2, *Propionibacterium acnes* T1 and UD, *T. denticola* ATCC 35405 and e′. Except for *T. denticola* and *B. forsythus*, all bacteria were grown in Todd Hewitt broth (BBL Microbiology Systems) supplemented with haemin (10 μg ml^{-1}) and vitamin K (1 μg ml^{-1}). *T. denticola* was cultured in the oral spirochaete medium as previously described by Leschine & Canale-Parola (1980), whereas *B. forsythus* was grown in Todd Hewitt broth supplemented with 5% (v/v) heat-inactivated calf serum and 0·001% (w/v) *N*-acetylmuramic acid. The cultures were incubated in an anaerobic chamber [$N_2/H_2/CO_2$ (80:10:10, by vol.)] at 37 °C to late exponential growth phase.

Isolation of bacterial proteases. Proteases from *P. gingivalis* and *T. denticola* were isolated by preparative PAGE according to a procedure previously described by Grenier (1992) and Uitto *et al.* (1988), respectively. A preparation of outer-membrane vesicles (Singh *et al.*, 1989) was used as starting material for the isolation of the 80 kDa protease from *P. gingivalis*. The 95 kDa protease from *T. denticola* was isolated from a sonicated cell extract (five times for 45 s, 30% duty cycle, output 5; Sonifier Cell Disrupter, Branson Sonic Power). The final protease preparations were adjusted to 35 and 60 μg protein ml^{-1} for *P. gingivalis* and *T. denticola*, respectively. Protein was quantified by using a Bio-Rad microassay with BSA as the standard. The presence of only one proteolytic enzyme in the final preparations was verified by SDS-PAGE incorporating covalently bound BSA (Grenier *et al.*, 1989). After electrophoresis, the gel was shaken in 0·1 M Tris/HCl, pH 7·2, containing 2% (v/v) Triton X-100, followed by a wash in 0·1 M Tris/HCl to remove the detergents. The gel was then incubated at 37 °C for 2 h in 0·1 M Tris/HCl, pH 7·2, supplemented with 10 mM DTT to allow the enzymic degradation of the conjugated protein substrate. The gel was then stained for proteins with Coomassie brilliant blue. Following destaining of the gel, the presence of a proteolytic activity was visualized as a clear band against a blue background. The molecular mass markers were myosin (200 kDa), phosphorylase b (97·4 kDa), BSA (69·8 kDa), ovalbumin (43 kDa) and carbonic anhydrase (29 kDa).

Assay for degradation of host protease inhibitors. The degradation of α-1-antitrypsin, antichymotrypsin, α_2-macroglobulin, antithrombin III, antiplasmin and cystatin C by

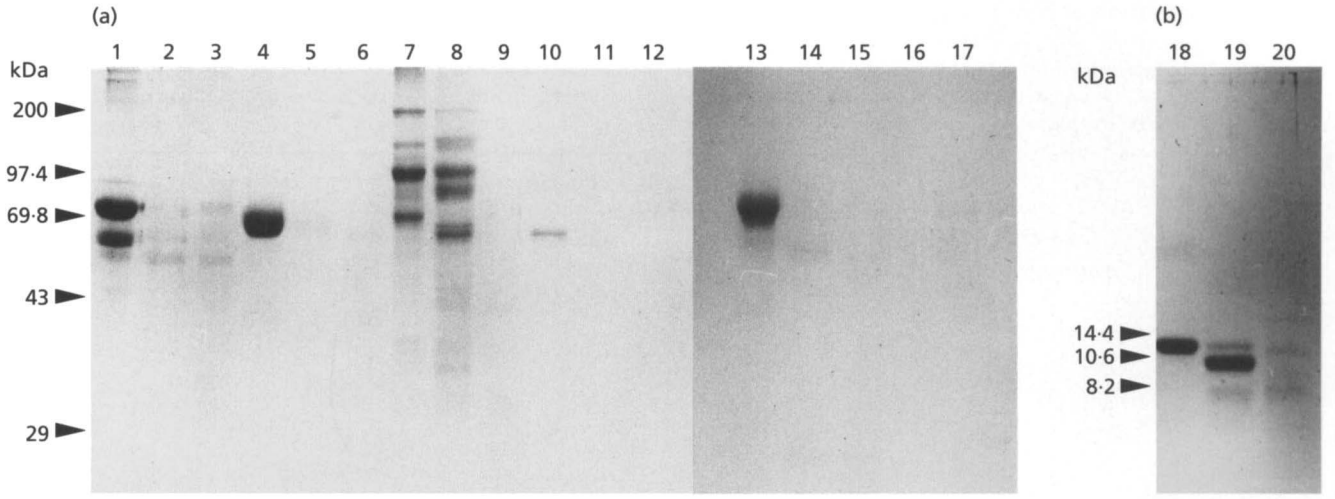

Fig. 2. Degradation of host protease inhibitors by the protease preparations. Inhibitors were incubated with the purified proteases (37 °C for 16 h), and the assay mixtures were boiled and analysed by SDS-PAGE. Lanes: 1–3, α-1-antitrypsin; 4–6, antichymotrypsin; 7–9, α_2-macroglobulin; 10–12, antithrombin III; 13–15, antiplasmin; 16–17, no inhibitor; 18–20, cystatin C. Assays in lanes 2, 5, 8, 11, 14, 16 and 19 were performed in the presence of the 95 kDa protease from *T. denticola*. Assays in lanes 3, 6, 9, 12, 15, 17 and 20 were performed in the presence of the 80 kDa protease from *P. gingivalis*. Molecular mass markers were (from top to bottom): myosin (200 kDa), phosphorylase b (97·4 kDa), BSA (69·8 kDa), ovalbumin (43 kDa) and carbonic anhydrase (29 kDa) for gel (a) and myoglobin I and II (14·4 kDa), myoglobin I and III (10·6 kDa) and myoglobin I (8·2 kDa) for gel (b). The high molecular mass band in the α-1-antitrypsin preparation is thought to represent transferrin contamination.

proteolytic enzymes and whole bacteria was determined by assaying for lower-molecular-mass degradation fragments by SDS-PAGE (11%, w/v, gel; 15% in the case of cystatin C) using the buffer system of Laemmli (1970). Briefly, 10 µl protease preparations or bacterial cells (OD_{660} = 2·0 in 50 mM Tris/HCl, pH 7·2) was incubated at 37 °C with 10 µl protease inhibitor (1 mg ml^{-1}) and with 20 µl 50 mM Tris/HCl, pH 7·2, containing 10 mM DTT. At the end of the incubation period (16 h for the protease preparations and 8 h for bacterial cells), cells were removed by centrifugation and 10 µl SDS sample buffer (0·125 M Tris/HCl, pH 6·8; 2%, w/v, SDS; 2%, w/v, mercaptoethanol; 20%, v/v, glycerol; 0·01%, w/v, bromophenol blue) was added prior to boiling for 10 min. Samples were then run on SDS-PAGE gels and proteins were stained with Coomassie brilliant blue.

Assay for plasminogen activator activity. Plasminogen activation was measured by mixing 20 µl serial dilutions (1:2) of the protease preparations with an equal volume of human plasminogen (2 mg ml^{-1}) and with 30 µl 50 mM Tris/HCl, pH 7·2. The human plasminogen and buffer were prereduced by an overnight incubation in the anaerobic chamber. The reaction mixtures were incubated anaerobically at 37 °C for 2 h. Thereafter, 15 µl Val-Leu-Lys-*p*-nitroanilide (2 mg ml^{-1}), a chromogenic substrate for human plasmin (Kulisek *et al.*, 1989), was added and the mixture was further incubated at 37 °C for 2 h prior to measuring the A_{405} with a microtitre plate ELISA reader (SLT-Labinstruments). Boiling the protease preparations or the omission of human plasminogen in the assay were used as controls. The assay was also carried out using streptokinase, a known plasminogen activator produced by most group A, C and G streptococci (Huang *et al.*, 1989). Following incubation of plasminogen with the protease preparations, a sample was obtained and analysed by SDS-PAGE (11% gel). The gel was stained for proteins using Coomassie brilliant blue.

RESULTS

Preparation of bacterial proteases

On an SDS-PAGE gel containing covalently bound BSA, a single proteolytic band was found in the final protease preparations (Fig. 1). The molecular mass of the *P. gingivalis* and *T. denticola* proteases was estimated to be 80 kDa (lane 2) and 95 kDa (lane 1), respectively. Using synthetic chromogenic peptides and specific protease inhibitors (data not shown), the proteases were found to have properties corresponding to those previously reported in the original studies (Grenier, 1992; Uitto *et al.*, 1988). The 80 kDa enzyme from *P. gingivalis* was a thiol-activated trypsin-like protease, whereas the 95 kDa enzyme from *T. denticola* was a thiol-activated chymotrypsin-like protease.

Degradation of protease inhibitors

Proteolytic breakdown of protease inhibitors by the two bacterial proteases was evaluated by SDS-PAGE (Fig. 2). The 80 kDa trypsin-like protease of *P. gingivalis* completely digested the six protease inhibitors under investigation (lanes 3, 6, 9, 12, 15 and 20). On the other hand, the 95 kDa chymotrypsin-like protease of *T. denticola* was found to degrade extensively α-1-antitrypsin (lane 2), antichymotrypsin (lane 5), antithrombin III (lane 11) and antiplasmin (lane 14). The protein band corresponding to the subunit of α_2-macroglobulin was only slightly decreased (lane 8). Finally, the *T. denticola* protease degraded

Table 1. Degradation of host protease inhibitors by periodontopathogens

Degradation was determined by SDS-PAGE. −, No degradation of the protease inhibitor band; +, partial degradation of the protease inhibitor band; + +, complete degradation of the protease inhibitor band; F, degradation with production of lower molecular mass fragments (number of fragments in parentheses).

Strain	Degradation					
	α-1-Antitrypsin	Antichymotrypsin	α_2-Macroglobulin	Antithrombin III	Antiplasmin	Cystatin C
A. actinomycetemcomitans						
ATCC 29522	−	−	−	−	−	−
Y4	−	−	−	−	−	−
B. forsythus						
ATCC 43037	−	−	−	−	−	−
4067M27	−	−	−	−	−	−
***Capnocytophaga* spp.**						
VA	−	F(1)	+	F(1)	F(3)	+
III.21	−	F(3)	+	F(2)	F(3)	F(1)
F. nucleatum						
102.3	−	−	−	−	−	−
CM33MB-5	−	−	−	−	−	−
Pept. micros						
NY370	−	−	+	−	−	+
89A	−	−	+	−	−	+
P. gingivalis						
ATCC 33277	+ +	F(1)	+ +	+ +	+ +	+ +
W50	+ +	F(1)	+ +	+ +	+ +	+ +
Prev. intermedia						
ATCC 25611	+	+	+	+ +	+	−
NY365	−	−	+	+	+	+
Prev. nigrescens						
NCTC 9336	+	+	+	+	F(2)	+
SPRO 2	−	+	+	+	+	+
Prop. acnes						
T1	−	−	−	+	−	F(1)
UD	−	−	−	+	−	−
T. denticola						
ATCC 35405	−	F(1)	+	+ +	F(2)	+
e′	−	+	+	+	F(2)	+

cystatin C with the production of one major fragment of less than 10 kDa (lane 19).

To investigate if other suspected periodontopathogens had the ability to degrade host protease inhibitors, similar assays of digestion were carried out using whole bacteria (Table 1). The most active bacteria were *P. gingivalis*, *T. denticola*, *Prev. intermedia*, *Prev. nigrescens* and *Capnocytophaga* spp. *Pept. micros* and *Prop. acnes* had only some degradative activity towards selected inhibitors. The complete disappearance of an inhibitor is related to a proteolytic degradation rather than to a binding to bacterial cells. This is indicated by the fact that no degradation was observed when heat-treated cells were used or when a cocktail of low molecular mass protease inhibitors was added to the assay mixtures (data not shown). Three bacterial species, *A. actinomycetemcomitans*, *B. forsythus* and *F. nucleatum*, had no effect on the host protease inhibitors tested.

Plasminogen activator activity

The ability of the protease preparations from *P. gingivalis* and *T. denticola* to activate human plasminogen is reported in Table 2. None of the proteases could degrade the plasmin substrate Val-Leu-Lys-*p*-nitroanilide. The 80 kDa protease of *P. gingivalis* demonstrated strong plasminogen activation, whereas no such activity was associated with the 95 kDa protease of *T. denticola*. Under the experimental conditions of the assay, the plasminogen activator activity found in the *P. gingivalis* protease

Table 2. Plasminogen activator activity of the 80 kDa trypsin-like protease from *P. gingivalis* and the 95 kDa chymotrypsin-like protease from *T. denticola*

Enzyme (dilution or amount)	Hydrolysis of plasmin substrate (A_{405})	
	With plasminogen	Without plasminogen
P. gingivalis **protease (80 kDa)**		
1:2	1·22	0·09
1:4	1·19	0·04
1:8	0·94	0·03
T. denticola **protease (95 kDa)**		
1:2	0·09	0·04
1:4	0·07	0·03
1:8	0·08	0·03
Streptokinase		
0·1 unit	1·31	0·04
0·05 unit	0·71	0·08
0·025 unit	0·29	0·03
0·0125 unit	0·16	0·03

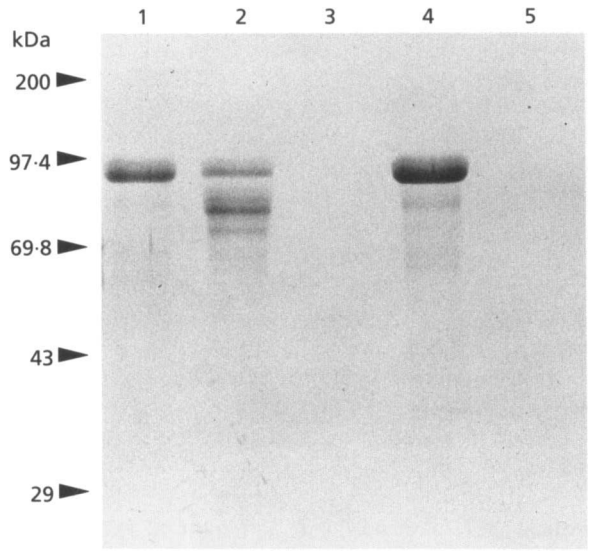

Fig. 3. Degradation of human plasminogen by the protease preparations. Plasminogen was incubated with the purified proteases (37 °C for 2 h), and the assay mixtures were boiled and analysed by SDS-PAGE. Lanes: 1, plasminogen alone; 2, plasminogen plus the 80 kDa protease from *P. gingivalis*; 3, 80 kDa protease from *P. gingivalis* alone; 4, plasminogen plus the 95 kDa protease from *T. denticola*; 5, 95 kDa protease from *T. denticola* alone. Molecular mass markers were (from top to bottom): myosin (200 kDa), phosphorylase b (97·4 kDa), BSA (69·8 kDa), ovalbumin (43 kDa) and carbonic anhydrase (29 kDa).

preparation corresponded to 28 units streptokinase ml^{-1}. SDS-PAGE analysis of the assay mixture showed that the *P. gingivalis* protease produced a number of fragments from plasminogen, including one with a molecular mass of 80 kDa which may represent the active form of plasmin (Fig. 3, lane 2).

DISCUSSION

The development of periodontal diseases is associated with an increased level of proteolytic activity in subgingival sites, including the gingival crevicular fluid (Eley & Cox, 1992; Sandholm, 1986). These enzymes, which originate from both the host and periodontopathogens, may participate in the pathogenesis of periodontal diseases through their action on host proteins. Several investigators have suggested that bacterial proteases may play a critical role in the development of periodontal diseases (Grenier & Mayrand, 1993; Holt & Bramanti, 1991). Since significant tissue damage is observed in periodontitis patients, it is thought that bacterial proteases may be, at least in part, responsible for this destruction. The fact that a significantly higher number of proteolytic bacteria was recently demonstrated in patients with periodontitis than in healthy patients reinforced this hypothesis (Grenier & Turgeon, 1994).

The direct destructive effect by *P. gingivalis* may involve collagenolytic enzymes previously characterized (Grenier & Mayrand, 1993). In the case of *T. denticola*, the 95 kDa chymotrypsin-like protease used in this study was reported to represent an important determinant in invasion of a basement membrane model (Grenier *et al.*, 1990). The indirect destructive effect by bacterial proteases may involve the digestion of host protease inhibitors. Plasma protease inhibitors present in gingival crevicular fluid as well as cystatin C, a tissue protease inhibitor present in inflamed gingiva, may play a critical role in the protection of periodontal tissues by modulating protease activity, more particularly during active phases. Indeed, protease inhibitors are involved in the regulation of various proteolytic cascade systems, such as the complement system, the kallikrein-kinin system and the fibrinolytic and clotting system, as well as inflammatory reactions. As this study demonstrated that most of these host protease inhibitors were susceptible to degradation by bacterial proteases, their protective effect may be reduced. Interestingly, the amount of plasma protease inhibitors and cystatin C was reported to decrease significantly with the progression of the disease (Lah *et al.*, 1993). This may result in an uncontrolled degradation of periodontal tissues and a rapid progression of the disease.

The ability of periodontopathic bacteria, more particularly *P. gingivalis* and *T. denticola*, to degrade and inactivate plasma protease inhibitors has been previously reported (Carlsson *et al.*, 1984; Fishburn *et al.*, 1991; Herrmann *et al.*, 1985; Nilsson *et al.*, 1985; Uitto *et al.*, 1988). As whole bacterial cells were used in most studies, the nature of proteases involved in the degradation was not identified. This report extends our knowledge by indicating that two cell-associated proteases, a trypsin-like protease (80 kDa) from *P. gingivalis* and a chymotrypsin-like protease (95 kDa) from *T. denticola*, may be, at least in part, responsible for the degradation. The most active

enzyme was found to be the 80 kDa protease from *P. gingivalis*, which completely degraded the six host protease inhibitors under investigation. Fishburn *et al.* (1991) previously reported that extracellular trypsin-like activity produced by *P. gingivalis* could hydrolyse the major serum proteins and could be inhibited by the serine protease inhibitor antithrombin III. In the present study, it was shown that the cell-associated 80 kDa trypsin-like protease of *P. gingivalis* completely digested antithrombin III.

A 35 kDa protease from *P. gingivalis* was previously found to stimulate fibroblasts to secrete plasminogen activator (Uitto *et al.*, 1989). Here, it is demonstrated that the 80 kDa protease from *P. gingivalis* activates human plasmin, a serine protease with trypsin-like activity. The fact that the 80 kDa protease of *P. gingivalis* does not degrade the plasmin substrate in the plasminogen activation assay indicates that it is a trypsin-like enzyme unable to cleave at bonds involving lysine residues. The active plasmin, if generated at subgingival sites, may be potentially deleterious as it is capable of activating the kinin cascade and is strongly implicated in the inflammatory process. It may also catalyse proteolytic cleavages in inactive zymogens of matrix metalloproteinases, leading to active enzymes (Mignatti *et al.*, 1986). Previous studies also indicated that a proteolytic fraction from *P. gingivalis* is able to activate latent matrix metalloproteinases (Birkedal-Hansen *et al.*, 1984) and induce their secretion by human gingival fibroblasts (Uitto *et al.*, 1989).

Additional pathogenic roles have been ascribed to the 80 kDa protease from *P. gingivalis*. This protease can inactivate the bactericidal activity of human serum, thus protecting susceptible bacteria found in periodontal pockets (Grenier, 1992). The 80 kDa protease can also generate Fc fragments from human IgG1 which can stimulate a marked increase in the release of interleukin-6, interleukin-8, and tumour necrosis factor alpha from human peripheral blood mononuclear cells and thus shows that this enzyme may contribute to the inflammatory process in periodontitis (Engel *et al.*, 1994).

In summary, this study is in agreement with the model of pathogenesis of periodontal disease proposed by Travis *et al.* (1994). It was suggested that following an overgrowth of *P. gingivalis*, the bacteria secreted proteinases which could not be neutralized by protease inhibitors found in subgingival sites. These bacterial proteases activate the complement system, resulting in a migration of inflammatory cells at the infection site. The inability of phagocytic cells to destroy bacteria results in a chronic influx of inflammatory cells associated with an important release of host hydrolytic enzymes, including proteases. Altogether, the host and bacterial enzymes will severely damage the periodontal tissues.

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