

Identification of Proteases from Periodontopathogenic Bacteria as Activators of Latent Human Neutrophil and Fibroblast-Type Interstitial Collagenases

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Activation of latent human fibroblast-type and neutrophil interstitial procollagenases as well as degradation of native type I collagen by supra- and subgingival dental plaque extracts, an 80-kDa trypsinlike protease from *Porphyromonas gingivalis* (ATCC 33277), a 95-kDa chymotrypsinlike protease from *Treponema denticola* (ATCC 29522), and selected bacterial species commonly isolated in periodontitis was studied. The bacteria included were *Prevotella intermedia* (ATCC 25261), *Prevotella buccae* (ES 57), *Prevotella oris* (ATCC 33573), *Porphyromonas endodontalis* (ES 54b), *Actinobacillus actinomycetemcomitans* (ATCC 29522), *Fusobacterium nucleatum* (ATCC 10953), *Mitsuokella dentalis* (DSM 3688), and *Streptococcus mitis* (ATCC 15909). None of the bacteria activated latent procollagenases; however, both sub- and supragingival dental plaque extracts (neutral salt extraction) and proteases isolated from cell extracts from potentially periodontopathogenic bacteria *P. gingivalis* and *T. denticola* were found to activate latent human fibroblast-type and neutrophil interstitial procollagenases. The fibroblast-type interstitial collagenase was more efficiently activated by bacterial proteases than the neutrophil counterpart, which instead preferred nonproteolytic activation by the oxidative agent hypochlorous acid. The proteases were not able to convert collagenase tissue inhibitor of metalloproteinase (TIMP-1) complexes into active form or to change the ability of TIMP-1 to inhibit interstitial collagenase. None of the studied bacteria, proteases from *P. gingivalis* and *T. denticola*, or extracts of supra- and subgingival dental plaque showed any significant collagenolytic activity. However, the proteases degraded native and denatured collagen fragments after cleavage by interstitial collagenase and gelatinase. Our results indicate that proteases from periodontopathogenic bacteria can act as direct proteolytic activators of human procollagenases and degrade collagen fragments. Thus, in concert with host enzymes the bacterial proteases may participate in periodontal tissue destruction.

Mammalian interstitial collagenases (EC 3.4.24.7, matrix metalloproteinases [MMPs] -1 and -8) are considered to be key initiators of collagen degradation during the progression of periodontal tissue destruction events (1, 4, 10, 22, 36, 37). Interaction of several plaque bacteria with inflammatory cells (neutrophils or monocyte/macrophages) and resident gingival fibroblasts results in protease release by the host cells (33, 36), and elevated levels of interstitial collagenases have been detected in inflamed human gingival tissue and gingival crevicular fluid, an inflammatory exudate of gingiva (22, 34, 36). Interstitial collagenase present in gingival tissue and crevicular fluid has been found to be converted from latent form to an actively destructive form of the enzyme in vivo by the inflammatory process of periodontal disease; this is probably mediated by the independent and/or cooperative action of activating host cell-derived proteinases and/or reactive oxygen species (3, 14, 17, 21, 37).

Porphyromonas gingivalis and *Treponema denticola* have been implicated as significant components of microbial flora in advanced periodontitis associated with tissue destruction (16, 18). The virulence of these microorganisms has been suggested to be related to several factors. Special attention has been focused on the strong proteolytic arsenal of *P. gingivalis* and *T. denticola* (11, 16, 18). Proteases of these

bacteria have been suggested to mediate tissue destruction events and to disturb the host defense mechanisms during periodontal inflammation (11). Even though both *P. gingivalis* and *T. denticola* have been found to produce several potent neutral proteases, only *P. gingivalis* has been demonstrated to synthesize true collagenase (2, 32, 36). However, direct evidence revealing the molecular mechanisms by which bacterial proteases participate in periodontal tissue destruction is still lacking.

Because of its relevance to *P. gingivalis*- and *T. denticola*-mediated periodontal tissue destruction, we explored the possibility that the proteases of these oral pathogens participate in collagen degradation either by activating latent interstitial collagenases or by preventing its inhibition by tissue inhibitor of metalloproteinases (TIMP-1). Interaction of several other bacteria present in periodontal infections and supra- and subgingival dental plaque extracts with latent interstitial collagenases and collagenase breakdown products was also studied.

MATERIALS AND METHODS

Purification of latent human neutrophil and fibroblast-type interstitial collagenases. Latent human neutrophil (proMMP-8) and gingival fibroblast (proMMP-1) interstitial collagenases were purified as described previously (12, 17, 33). Briefly, gingival fibroblasts (33) were planted on tissue culture cham-

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ber slides (grown area, 1.8 cm² per chamber; Lab-Tek 4804, Miles Scientific, Division of Miles Laboratories, Naperville, Ill.) to a final volume of 1.0 ml. Fibroblast culture medium was supplemented for 24 h either with purified interleukin-1 (Gene-enzyme/Koch-Light, Suffolk, United Kingdom) to a final concentration of 1 IU/ml or with recombinant interleukin-1 β (Collaborative Research Incorporation, Bedford, Mass.) at 1 or 5 half-maximal units/ml (12). Supernatants were assayed for interstitial collagenase activity (see below). Human gingival fibroblast (proMMP-1) interstitial procollagenase was purified to apparent homogeneity by the column chromatographic method of gel filtration on Sephacryl S-200, ion-exchange chromatography on carboxymethyl cellulose-Sepharose, and affinity chromatography on Cibacron Blue-Sepharose. Latent 70- to 75-kDa human neutrophil interstitial collagenase was purified to apparent homogeneity from neutral salt extracts of human neutrophils by using the corresponding column chromatographic protocol, except that a QAE-Sepharose instead of carboxymethyl cellulose-Sepharose ion-exchange column was used (12, 17). The purified fibroblast-type and neutrophil interstitial (pro)collagenases did not exert activity against heat-denatured type I collagen, indicating that the enzymes were free of type IV/gelatinase and stromelysin-like activities (12, 17).

Bacterial cultures and isolation of proteases from *P. gingivalis* and *T. denticola*. Bacteria obtained from reference culture collections and our own strains isolated from endodontic and periodontic infections were used for the experiments (6–9, 15, 32). The strains were preserved deep frozen in glycerine-milk at –70°C and maintained for this study by weekly transfer on Brucella horse blood agar supplemented with menadione (0.5 μ g/ml), hemin (5 μ g/ml), and cysteine (750 μ g/ml). For the experiments the bacteria (Fig. 1 and 2; Tables 1 and 2 [6–9, 15, 32]) were grown in Mycoplasma broth medium (15) supplemented with menadione (0.5 μ g/ml), hemin (5 μ l/ml), and cysteine (750 μ g/ml). *Actinobacillus actinomycetemcomitans* was grown in Todd-Hewitt broth. *T. denticola* was grown in a special broth (9); 100 ml of this broth contained the following: heart infusion broth, 1.25 g (Difco Laboratories, Detroit, Mich.); Trypticase, 1 g (BBL Microbiology Systems, Cockeysville, Md.); yeast extract, 0.25 g (Difco); sodium thioglycolate, 0.05 g (Difco); L-cysteine hydrochloride, 0.1 g (Sigma Chemical Co., St. Louis, Mo.); L-asparagine, 0.025 g (Sigma); glucose, 0.2 g (Difco); thiamine PP₁, 0.6 mg (Sigma); isobutyric acid, 0.001% (vol/vol; Sigma); valeric acid, 0.001% (vol/vol; Sigma); sodium bicarbonate, 0.2% (wt/vol; Sigma); and heat-inactivated horse serum, 2 ml (Cibco, Burlington, Ontario, Canada). Three-day-old cultures were collected by centrifugation (10,000 \times g), washed once in phosphate-buffered saline, and used for the experiments. In some experiments bacterial sonicates instead of whole cells were used. Briefly, the bacteria were suspended in 50 mM Tris-HCl–0.2 M NaCl–5 mM CaCl₂ (TNC buffer), pH 7.5, at a concentration of 20 mg/ml (wet weight) of bacteria. The samples were sonicated on ice six times for 10 s each, and centrifuged at 15,000 \times g for 30 min. The supernatants were used for experiments (32).

The 80-kDa trypsinlike protease from *P. gingivalis* and 95-kDa chymotrypsinlike protease from *T. denticola* were isolated from cell extracts of these bacteria as described elsewhere in detail (5, 31). Briefly, both bacterial proteases were purified to essential homogeneity by an isolation protocol consisting of preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroelution of the proteases from the gels (5, 31). The isolated proteases were extensively dialyzed against TNC buffer in

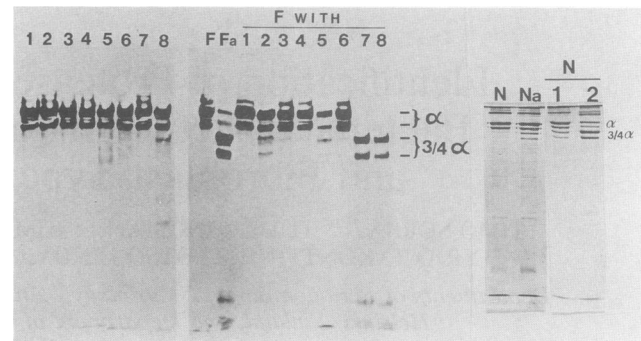


FIG. 1. Activation of latent fibroblast and neutrophil procollagenases by periodontopathogenic bacteria and proteases from *T. denticola* and *P. gingivalis*. Type I collagen and human fibroblast and neutrophil procollagenases (5 μ g/ml [12, 17, 33]) were incubated in the presence of 10⁷ whole bacterial cells or 10 μ g of purified *T. denticola* and *P. gingivalis* proteases per ml (5, 31). Lane 1, *Prevotella intermedia* (ATCC 25261); lane 2, *P. gingivalis* 80-kDa protease (5); lane 3, *A. actinomycetemcomitans* (ATCC 295222); lane 4, *Fusobacterium nucleatum* (ATCC 10953); lane 5, *T. denticola* 95-kDa protease (31); lane 6, *Streptococcus mitis* (ATCC 15909); lane 7, neutral salt extract of supragingival plaque; lane 8, extract of subgingival plaque. F, fibroblast collagenase; Fa, fibroblast procollagenase activated with 1 mM APMA (17); N, neutrophil procollagenase; Na, neutrophil procollagenase activated with 1 mM APMA. Lane 1, *T. denticola* 95-kDa protease pretreatment; lane 2, *P. gingivalis* 80-kDa protease pretreatment. The samples underneath F and N are bacterial samples incubated in the presence of the fibroblast-type (F) and neutrophil (N) procollagenases. Intact collagen α -chains and their 3/4-cleavage fragments produced by active mammalian collagenases have been denoted as α and 3/4 α , respectively. Note that none of the bacteria, the studied proteases, and the dental plaque extracts had significant collagenolytic activity. The proteases from *T. denticola* and *P. gingivalis* as well as dental plaque extracts, however, activated latent collagenases.

order to obtain the enzymes free from SDS. Purified TIMP-1 was kindly provided by Tim Cawston, Addenbrooks Hospital, Cambridge, United Kingdom.

The whole supra- and subgingival dental plaque samples were obtained from patients with adult periodontal disease (25). After the dental plaque samples were washed three times with TNC buffer, the dental plaque mass was suspended in TNC buffer at 10 mg/ml (34). Subsequently, the plaque sample was disrupted by freezing and thawing three times and by sonication as described above for cultured bacteria. The recovered supernatants were used at a 1/10 dilution in the experiments (34).

Activation of latent human interstitial collagenases and assay of collagenase and gelatinase activities. Latent human MMPs (5 μ g of enzyme protein per ml) were treated with known nonproteolytic activators, 1 mM aminophenylmercuric acetate (APMA; an optimal organomercurial activator of proMMPs [12, 17, 33], Sigma), and 1 mM hypochlorous acid (NaOCl, an oxidative activator [12, 13, 17], purchased from the Pharmacy of the University of Helsinki, Helsinki, Finland), and pancreatic trypsin (5 μ g/ml; Sigma) to ensure optimal activation of proMMPs as described previously (3, 12, 14, 17, 33). In the activation experiments with bacterial proteases latent proMMPs (5 μ g of enzyme protein per ml) were incubated with *P. gingivalis* and *T. denticola* proteases (10 μ g/ml) for 1 h at 37°C before inactivation of the bacterial proteases by 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma) and 5 mM benzamidine (EGA-Chemie) (5, 31).

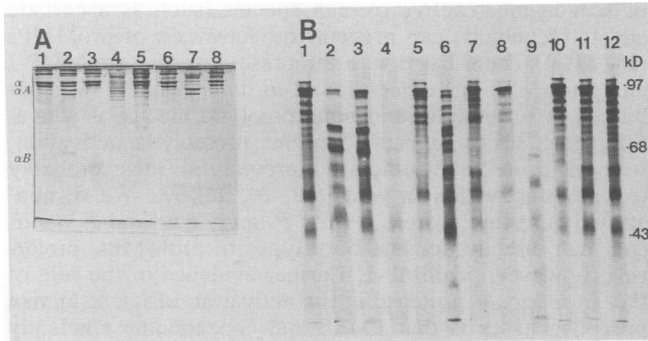


FIG. 2. (A) Effects of serine protease inhibitors on the activation of latent human neutrophil procollagenase (MMP-8) by the *T. denticola* 95-kDa protease. Lane 1, type I collagen incubated with proMMP-8 (5 µg of enzyme protein per ml); lane 2, same as lane 1 but with 1 mM APMA activation; lane 3, type I collagen incubated with buffer; lane 4, same as lane 1 but in the presence of *T. denticola* protease (10 µg/ml); lane 5, same as lane 4 but in the presence of 1 mM PMSF and 5 mM benzamidine. In this experiment (lanes 4 and 5), prolonged incubation (18 h at 22°C) of proMMP-8 and *T. denticola* protease was used instead of the 1-h incubation used in typical activation experiments (Fig. 1 and Table 1 as well as the following lanes 6 to 8). Lane 6, same as lane 1; lane 7, same as lane 4 but with a 1-h incubation; lane 8, same as lane 7 but in the presence of 1 mM PMSF and 5mM benzamidine. Intact type collagen α-chains and their 3/4- and 1/4-cleavage fragments produced by active mammalian interstitial collagenase have been denoted as α and αA and αB, respectively. Note that *T. denticola* protease activated proMMP-8 and concomitantly further degraded the αA-chains. Both these effects could be prevented by the serine protease inhibitors. (B) Degradation of denatured collagen fragments by oral bacteria. Soluble ¹⁴C-labelled denatured type I collagen was autolyzed by prolonged incubation at 42°C. Sonicated bacterium extracts (20 µg of protein) were incubated at 37°C with denatured collagen fragments for 1 h. The samples were then subjected to SDS-PAGE and fluorography (32). Lane 1, control; lane 2, *P. intermedia* (ATCC 25261); lane 3, *Porphyromonas endodontalis* (ES 54b); lane 4, *P. gingivalis* (ATCC 33277); lane 5, *Prevotella buccae* (ES 57); lane 6, *Prevotella oris* (ATCC 33573); lane 7, *Mitsukoella dentalis* (DSM 3688); lane 8, *Prevotella buccae* (ATCC 33690); lane 9, *T. denticola* (ATCC 29522); lane 10, *A. actinomycetemcomitans* (Y4); lane 11, *A. actinomycetemcomitans* (ATCC 29522); lane 12, *F. nucleatum* (ATCC 10953). Molecular mass markers are indicated.

Procollagenases were also incubated in the presence of the indicated whole bacterial cells (10⁷/ml) as well as extracts of supra- and subgingival dental plaque. Subsequently, the samples were incubated with native soluble 1.5 µM type I collagen at 22°C for the indicated times. In separate incubations, type I collagen was incubated with the studied *P. gingivalis* and *T. denticola* proteases and the indicated whole bacterial cells as well as neutral salt extracts of supra- and subgingival dental plaque. To study whether the bacterial proteases degraded collagen fragments, after their denaturation, peptides of different sizes were produced by incubating heat-denatured ¹⁴C-labelled type I collagen at 40°C for 48 h. Small amounts of the gelatinase that was bound to the collagen fibrils degraded them to several fragments. The fragments were then heated at 90°C for 60 min to inactivate gelatinase. The peptides were then incubated at 37°C for 1 h with the indicated bacterial extracts. Incubations were stopped by the addition of Laemmli's sample buffer containing 40 mM EDTA and then by immediate heating at 100°C. The specific degradation products of type I collagen resulting from mammalian interstitial collagenase action as well as

TABLE 1. Activation of human fibroblast-type (MMP-1) and neutrophil (MMP-8) procollagenases by nonproteolytic and proteolytic means^a

Treatment	% Degradation	
	MMP-1	MMP-8
Buffer	8	12
1 mM APMA	97	92
1 mM NaOCl	22	95
Trypsin (5 µg/ml)	87	34
<i>P. gingivalis</i> 80-kDa protease (10 µg/ml)	64	32
<i>T. denticola</i> 95-kDa protease (10 µg/ml)	45	52

^a Human fibroblast-type (MMP-1) and neutrophil (MMP-8) interstitial procollagenases (5 µg of enzyme protein per ml) were treated with buffer, 1 mM APMA, 1 mM NaOCl, pancreatic trypsin (Sigma), *P. gingivalis* 80-kDa protease, and *T. denticola* protease as described in the text; protease activation incubations were stopped by 1 mM PMSF and 5 mM benzamidine. Subsequently mixtures were incubated with soluble 1.5 µM type I collagen for 18 h at 22°C. Collagenase activity was measured by quantitative SDS-PAGE assay. Values are percent type I collagen degraded and are means of three determinations.

gelatin peptides were separated by SDS-PAGE on 8 or 10% cross-linked gels and processed for fluorography (in the case of radioactive substrate) or stained with Coomassie brilliant blue and destained in 5% methanol–10% acetic acid (nonlabelled substrate) (12, 17, 32). The fluorographs and gels were quantitated by densitometric scannings with an LKB Ultrascan Laser Densitometer, model 2202. The value representing αA-chains was multiplied by 4/3, and its proportion of total collagen in the sample was used as measure of mammalian interstitial collagenase activity. Collagenase activity is expressed as the percentage of type I collagen degraded (12, 17, 32). In the experiments on the abilities of the bacteria to degrade TIMP-1-collagenase complexes, autoactive human MMP-1 was treated with human TIMP-1 in an approximated molar ratio of 1:1 for 1 h at 37°C to ensure complete inactivation of MMP-1. Samples were then incubated with

TABLE 2. Effects of oral bacteria on the activity of TIMP-1 and human collagenase

Sample	% Collagen degraded
Buffer	8
Collagenase	78
Collagenase + TIMP-1	11
Collagenase + TIMP-1 + <i>P. intermediums</i>	10
Collagenase + TIMP-1 + <i>P. endodontalis</i>	12
Collagenase + TIMP-1 + <i>P. gingivalis</i> 80-kDa protease	22
Collagenase + TIMP-1 + <i>T. denticola</i> 95-kDa protease	18
Collagenase + TIMP-1 + <i>A. actinomycetemcomitans</i>	7
Collagenase + <i>P. intermediums</i>	75
Collagenase + <i>P. endodontalis</i>	81
Collagenase + <i>P. gingivalis</i> 80-kDa protease	84
Collagenase + <i>T. denticola</i> 95-kDa protease	78
Collagenase + <i>A. actinomycetemcomitans</i>	75

^a Autoactive fibroblast-type interstitial collagenase (5 µg/ml; MMP-1) was incubated with TIMP-1 in an approximated molar ratio of 1:1 for 1 h at 37°C, and then incubated with sonicated bacterial extracts (20 µg of protein) or purified *P. gingivalis* 80-kDa protease and *T. denticola* 95-kDa protease (10 µg/ml). Soluble type I [¹⁴C]collagen was then added, and the mixture was incubated for 18 h at 22°C. The values have been calculated from densitometric scans of SDS-PAGE fluorographs (32) and are means of three determinations. Note that none of the bacterial samples were able to reverse the TIMP-1-mediated inhibition of collagenase or inhibit the fibroblast collagenase activity.

bacterial extracts for 1 h at 37°C. In other experiments TIMP-1 was first preincubated with bacterial extracts and then with collagenase. Subsequently the samples were assayed for collagenase activity as described above.

RESULTS

To investigate whether protease or other substances from oral bacteria are able to activate latent human interstitial procollagenases, procollagenases from human neutrophils (proMMP-8) and fibroblasts (proMMP-1) were incubated in the presence of whole bacteria, isolated bacterial proteases, or neutral salt extracts of dental plaque. Degradation of native type I collagen was then assayed. None of the studied bacterial species, bacterial proteases (*P. gingivalis* 80-kDa protease and *T. denticola* 95-kDa protease [5, 31]), or extracts of supra- and subgingival dental plaque had any significant collagenolytic activity (Fig. 1, lanes 1 to 8). Pretreatment of procollagenases with *P. gingivalis* 80-kDa trypsinlike and *T. denticola* 95-kDa chymotrypsinlike proteases resulted in partial activation of both types of proMMPs (Table 1 and Fig. 1, lanes F2, F5, N1, and N2). In addition, supra- and subgingival plaque extracts fully activated procollagenase (Fig. 1, lanes F7 and F8). The results demonstrated that fibroblast-type collagenase (proMMP-1) preferred the proteolytic activation either by commercial pancreatic trypsin or by bacterial proteases when compared with the activation achieved by the oxidative agent NaOCl, whereas neutrophil collagenase (proMMP-8) was found to prefer the oxidative activation by NaOCl over the proteolytic activation provided either by pancreatic trypsin or by bacterial proteases (Table 1). The protease from *T. denticola* was also found to be capable of degrading the characteristic collagen cleavage products (α A chains) resulting from the action of collagenase concomitant with activation of proMMP-8; this effect could be prevented by 1 mM PMSF and 5 mM benzamidine (noncompetitive and competitive serine protease inhibitors, respectively) (Fig. 2A, lanes 1 to 8). In addition, bacterial proteases were able to degrade denatured collagen peptides, although different rates and patterns of degradation were noted (Fig. 2B, lanes 1 to 12). Bacterial proteases were not able to reactivate collagenase-TIMP-1 complex, nor were they able to reduce the capacity of TIMP-1 to inhibit collagenase (Table 2).

DISCUSSION

During the inflammatory process associated with periodontal diseases, procollagenases (either proMMP-1 or proMMP-8) are known to be converted to catalytically active form (22, 34, 36). We have recently found that in distinct forms of periodontal diseases, i.e., adult, juvenile, and diabetes-associated periodontitis, proMMP-1 and proMMP-8 seem to have specific roles in periodontal tissue destruction (19, 23, 25, 27–30, 35). Several mechanisms for the activation of latent proMMPs *in vitro* have been demonstrated, including activation by host cell-derived proteases such as trypsin/tryptases, stromelysin, and cathepsin G (3, 14, 17, 20). Also, nonproteolytic means of activation including compounds capable of modifying protein sulfhydryl groups, such as organomercurials, gold (I) compounds, and reactive oxygen species, have been described (3, 12, 13, 17, 20, 21, 26, 37). Of these nonproteolytic means of activation of proMMPs, reactive oxygen species generated by neutrophils may play a significant role during inflammatory processes *in vivo* (19, 20, 37). Furthermore, compounds capable

of scavenging reactive oxygen species (such as ascorbate and tetracyclines) can prevent the activation of proMMPs (13, 29). In accordance with the present results, proMMP-1 and proMMP-8 have been found to differ in their susceptibilities to proteolytic and nonproteolytic means of activation; proMMP-1 appears to prefer proteolytic activation, whereas proMMP-8 prefers nonproteolytic, most probably oxidative activation *in vivo*. (19, 20, 26, 37). We demonstrated here that proteases from *P. gingivalis* and *T. denticola* can directly activate both types of proMMPs, preferring, however, proMMP-1. Further evidence of the role of the *T. denticola* protease in the activation of latent human procollagenases is that PMSF and benzamidine efficiently inhibited the activation of latent proMMP-8 by the *T. denticola* protease. The bacterial proteases were not able to reactivate collagenase bound to TIMP-1 or alter the inhibitory potential of TIMP-1, indicating an unusual resistance of the TIMP-1 structure to bacterial proteolytic enzymes that is probably due to the stable disulfide-mediated cross-link structure of the molecule (38). The possible role of these bacteria in enhancing the activity of mammalian interstitial collagenases appears to be direct activation of procollagenases and completion of the action of mammalian collagenases by further degrading collagen and gelatin fragments. Of additional relevance to these observations is that we have previously found that a protease from *P. gingivalis* capable of degrading connective tissue and cell surface proteins can induce cultured gingival fibroblasts to secrete proMMP-1 and plasminogen activator (24, 33). Thus, proteases from potent periodontopathogenic bacteria can act as direct activators of proMMPs and induce their production by host cells as well as degrade efficiently collagen peptides after collagenase and gelatinase cleavages. These findings may explain a part of the molecular mechanisms of the destructive inflammation involved in periodontal tissue destruction induced and maintained by periodontopathogenic bacteria.

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