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Porphyromonas gingivalis affects host collagen degradation by affecting expression, activation, and inhibition of matrix metalloproteinases

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Objective: Studies have shown that *Porphyromonas gingivalis* and host matrix metalloproteinases (MMPs) play important roles in the tissue destruction associated with periodontal disease. It is still unclear which MMPs or their inhibitors are regulated by *P. gingivalis* at the transcriptional and/or at the protein levels. Therefore, this study was conducted to determine what effects *P. gingivalis* supernatant has on the collagen degrading ability of human gingival fibroblasts (HGFs) and how it regulates the activation, mRNA expression, and inhibition of MMPs.

Methods: Culture supernatant from *P. gingivalis* ATCC 33277 was added to HGFs cultured in six-well plates coated with Type I collagen. At certain time intervals, the cell conditioned media was collected for zymography and/or western blot analyses to determine the MMP and tissue inhibitor of MMPs (TIMP) protein levels. The cells were then removed and the collagen cleavage visualized by Coomassie blue staining. The mRNA expression of multiple MMPs and TIMPs by the treated and untreated HGFs was determined by reverse transcription–polymerase chain reaction.

Results: The collagen in the six-well plates was degraded more rapidly by the HGFs treated with 10% v/v *P. gingivalis* supernatant. More active MMP-1, MMP-2, MMP-3, and MMP-14 were detected in the conditioned media from the HGFs treated with the *P. gingivalis* supernatant. TIMP-1, but not TIMP-2, was decreased in the presence of the *P. gingivalis* supernatant. MMP-1 mRNA expression by the treated HGFs increased more than two-fold over the untreated HGFs. MMP-3 mRNA was unchanged, MMP-2 mRNA had a slight increase, MMP-14 mRNA decreased, and MMP-15 increased. MMP-12 mRNA was induced in the *P. gingivalis* treated HGFs. TIMP-1 and TIMP-2 mRNA had a slight increase with *P. gingivalis* treatment.

Conclusion: Porphyromonas gingivalis increased the collagen degrading ability of HGFs, in part, by increasing MMP activation and by lowering the TIMP-1 protein level, as well as by affecting the mRNA expression of multiple MMPs and TIMPs.

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Periodontal disease has been recognized to be due, in part, to the altered regulation of proteolytic activity. Matrix metalloproteinases (MMPs) are a group of zinc-dependent endopeptidases that include collagenases, gelatinases, stromelysins, membrane-associated MMPs, and other MMPs (1). MMPs play important roles in the turnover of connective tissue in both physiological and pathological conditions (2). Several members of the MMP family have been shown to be involved in periodontal tissue destruction and these include MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, and MMP-13 (2–10). Tissue inhibitors of MMPs (TIMPs) are important endogenous inhibitors of the MMPs (11). They are a family of low molecular mass inhibitors secreted by various cell types and are found in most body fluids and tissues. They bind specifically to the active sites of the MMPs and maintain the equilibrium between synthesis and degradation of the extracellular matrix (11, 12).

Studies have shown that host collagen degradation and some MMP levels are regulated by periodontal bacteria and their products. Dental plaque extracts have been observed to be able to influence the proteinases released from host cells (13) and to activate human fibroblast-type and neutrophil procollagenases (5). *Porphyromonas gingivalis* is regarded as one of the major periodontal pathogens (14). It is a Gram-negative, anaerobic, non-motile, and non-sporulating coccobacillus (15). These bacteria produce various virulence factors that allow them to survive in the hostile environment of the gingival sulcus or the periodontal pocket by evading the host antimicrobial defenses.

Porphyromonas gingivalis has been reported to affect the release and activation of certain MMPs from periodontal fibroblasts (16–19). Live *P. gingivalis* bacteria (5, 20), sonicated *P. gingivalis* bacterial extracts (21), and *P. gingivalis* outer membrane extracts (20) can activate certain pro-MMPs and/or regulate their mRNA expression levels. *Porphyromonas gingivalis* supernatant, which contains numerous virulence factors, has been reported to stimulate epithelial and foreskin fibroblast cells to degrade an underlying collagen Type I fibril bed (16, 22) and to increase the expression and activation of certain MMPs from the host cells (17, 18, 23). Nevertheless, it is still not clear which MMPs are regulated by these bacteria at the level of protein activation and at the level of mRNA expression, as well as how the TIMPs are affected. It has been demonstrated that the mRNA of some MMPs such as MMP-1, MMP-3, and MMP-14 are up-regulated by *P. gingivalis* (18, 23), yet the mRNA profiles for these and other MMPs in human gingival fibroblasts (HGFs) in the presence of *P. gingivalis* have not yet been examined.

Therefore, this study was conducted to determine the effects that *P. gingivalis* supernatant has on the collagen degrading ability of HGFs and on the expression of multiple MMPs and TIMPs at the protein and mRNA levels. This study extends our understanding of the role(s) that MMPs, TIMPs, and *P. gingivalis* play in the pathogenesis of periodontal disease.

Materials and methods

Cell culture

HGFs were cultured from explants of clinically healthy gingival connective tissue removed from patients undergoing crown lengthening surgery at Indiana University School of Dentistry with Institutional Review Board approval. The tissue was transported from the clinic to the laboratory in phosphate-buffered saline solution, washed with 70% ethanol, and rinsed in phosphate-buffered saline to remove the ethanol. The washing and rinsing steps were repeated and then the tissue was minced into small fragments of approximately 1 mm^3. The tissue pieces were then placed in cell culture dishes, air-dried, and incubated for 5–7 days at 37°C and 5% CO_2 in low glucose (1 g/l) Dulbecco's modified Eagle's (DME) media (Hyclone, Logan, UT, USA) supplemented with 15% fetal bovine serum, 4 mM L-glutamine, 100 U/ml penicillin, 50 μg/ml gentamicin, and 2.5 μg/ml fungizone. The cells that grew out of the explants were subcultured and maintained. Cells at passage 3–8 were utilized in the experiments.

Bacterial growth and the collection of the culture supernatant

The *P. gingivalis* ATCC 33277 supernatant was provided as a gift from Dr M. Lantz (Michigan University School of Dentistry, Ann Arbor, MI, USA). *Porphyromonas gingivalis* ATCC 33277 was maintained on enriched trypticase soy agar plates containing 3% sheep blood. Cultures were grown in brain heart infusion broth containing 1 mg/ml of cysteine, 5 μg/ml of hemin, and 0.5 μg/ml of menadione (supplemented brain heart infusion broth). Cultures were incubated in an anaerobic chamber (Coy Laboratories, Ann Arbor, MI, USA) with an atmosphere of 85% nitrogen, 10% hydrogen, and 5% carbon dioxide. When the bacterial growth yielded an OD_{660} of 1.0–1.2, the culture supernatant was harvested by centrifugation at 13,000 *g* for 20 min at 4°C. The collected supernatant was filtered twice through 0.2 μm membranes and then stored at −20°C.

Collagen degradation

The collagen degrading ability of HGFs was determined with a reconstituted collagen Type I assay system previously described by Birkedal-Hansen *et al.* (22). Briefly, rat tail tendon Type I collagen was dissolved in 13 mM HCl and then mixed rapidly on ice with one-fifth volume of a neutralizing phosphate buffer (40 ml of 0.2 M NaH_2PO_4/Na_2HPO_4 buffer, pH 7.4, 40 ml 0.1 M NaOH, 8.3 ml 5 M NaCl) to yield a final collagen concentration of 300 μg/ml. Aliquots of 1.5 ml/well (450 μg collagen/well) were dispensed in six-well plates and incubated at 37°C for 2 h. The collagen gels were dehydrated overnight in a laminar flow hood, washed three times for 30 min with sterile water, and then air-dried in the hood. HGFs were detached with 0.25% trypsin (Invitrogen, Carlsbad, CA, USA), pelleted, resuspended in serum-free DME medium, and then seeded as single colonies (50,000 cells/well)

in the center of six-well plates coated with collagen. After the cells attached, 2 ml serum-free DME medium containing different dilutions (1–10% v/v) of *P. gingivalis* supernatant was added to each well. Serum-free medium, medium containing 10% bacteria growth media, and medium containing 10% denatured *P. gingivalis* supernatant (by boiling at 100°C for 15 min) were utilized as experimental controls. After certain experimental periods (1–6 days), the conditioned media from the HGF cells was collected for zymography before the cells were removed with 500 μl of 0.1% Triton X-100 and 200 μl of 0.25% trypsin (Invitrogen). The wells were then stained with Coomassie blue to visualize the collagen cleavage. The experiments were repeated three or more times.

Gelatin zymography

The conditioned HGF media was mixed with non-reducing loading buffer (without β-mercaptoethanol) and resolved at 200 V in 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) gels copolymerized with 1 mg/ml gelatin. The gels were then washed with solution 1 (50 mM Tris, pH 7.5, 3 mM NaN_3, 2.5% Triton X-100), solution 2 (50 mM Tris, pH 7.5, 3 mM NaN_3, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 2.5% Triton X-100), and solution 3 (50 mM Tris, pH 7.5, 3 mM NaN_3, 5 mM $CaCl_2$, 1 μM $ZnCl_2$) for 20 min each. The gels were then incubated in fresh solution 3 at 37°C overnight and stained with Coomassie blue to visualize the proteolytic bands. The experiments were repeated three or more times.

Western blot analyses

HGFs were cultured for 48 h with or without 10% v/v *P. gingivalis* supernatant. The cell conditioned media was collected and concentrated 16-fold with a Centricon-10 (Millipore, Bedford, MA, USA). The concentration of the total protein in the concentrated media was determined according to the Bio-Rad Protein Assay protocol (Bio-Rad Laboratories, Hercules, CA, USA). The same amount of total protein of untreated and *P. gingivalis* treated concentrated media was resolved in 10% SDS–PAGE gels at 200 V. The proteins on the gels were transferred to nitrocellulose membranes at 0.3 A for 1 h using blotting buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, and 10% methanol). The membranes were then incubated in 5% milk in phosphate-buffered saline solution with 0.1% Tween-20 (pH 7.4) for 1 h to block non-specific binding. The membranes were then incubated with primary antibodies at 4°C for overnight. Antibodies used in this study were monoclonal Ab-5 (5 μg/ml, Clone III12b, NeoMarkers, Fremont, CA, USA) for MMP-1, monoclonal Ab-2 (5 μg/ml, Clone VB3, NeoMarkers) for MMP-2, monoclonal Ab-2 (5 μg/ml, Clone IID4, NeoMarkers) for MMP-3, polyclonal Ab 815 (0.3 μg/ml, Chemicon, Temecula, CA, USA) for MMP-14, polyclonal Ab 2315 (5 μg/ml, a gift from Dr K. Bodden, Mobile, AL, USA) for TIMP-1, and monoclonal Ab-2 (5 μg/ml, Clone IC3, NeoMarkers) for TIMP-2. The membranes were then washed three times with phosphate-buffered saline with Tween and incubated with anti-mouse or anti-rabbit secondary antibodies (Amersham, Piscastaway, NJ, USA) for 1 h at room temperature. The membranes were then developed with the ECL™ kit (Amersham) according to the manufacturer's protocol. The western blot analyses for each MMP and TIMP were repeated three to five times.

Direct degradation of the tissue inhibitors of matrix metalloproteinases by *Porphyromonas gingivalis* supernatant

The ability of *P. gingivalis* supernatant to digest TIMP-1 and TIMP-2 was determined. TIMP-1 was purified as described by Bodden *et al.* (24). Recombinant human TIMP-2 was a gift from Dr H. Birkedal-Hansen (Bethesda, MD, USA). The TIMPs (10 μg/ml) were incubated with or without 10% v/v *P. gingivalis* supernatant at 37°C for 0–24 h in 50 mM Tris, pH 7.4, 0.2 M NaCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, with 15 mM NaN_3. To determine the role of the cysteine proteinases (gingipains) in the *P. gingivalis* supernatant in this degradation, the cysteine proteinase inhibitor E64 (L-3-carboxy-2,3-*trans*-epoxypropionyl-leucylamido(4-guanidino)-butane, 0.1 mM) was pre-incubated with the *P. gingivalis* supernatant for 15 min at 37°C and then added to TIMP-1 (10 μg/ml). It was then incubated at 37°C for 0–24 h. The samples were added to 2 × electrophoresis loading dye (with β-mercaptoethanol), resolved in 15% SDS–PAGE gels by electrophoresis at 200 V, and then blotted to nitrocellulose membranes for western blot analyses. The experiments were repeated three or more times.

Reverse transcription–polymerase chain reaction

To determine the mRNA levels of multiple MMPs and TIMPs expressed by HGFs treated with or without 10% *P. gingivalis* supernatant for 48 h, total RNA was extracted using the Qiagen RNeasy mini kit (Qiagen, Valencia, CA, USA). Polymerase chain reaction (PCR) primers as previously described (25, 26) and mRNA were mixed with the Qiagen OneStep reverse transcription (RT)–PCR mix (Qiagen) according to the manufacturer's protocol. RT–PCR was performed using a Bio-Rad iCycler (Bio-Rad Laboratories). Reverse transcription was accomplished at 50°C for 30 min and then terminated at 95°C for 15 min. PCR was then performed for 30 cycles with denaturation at 95°C, annealing at 50°C, and extension at 72°C for 1 min each. The RT–PCR products were then resolved in 1% (w/v) agarose gels at 120 V, stained with ethidium bromide, and photographed under ultraviolet light. For estimation of the PCR results, the photographs obtained from transillumination were scanned and analyzed by NIH Scion Image program (Version 1.62). At cycle 30, the RT–PCR products produced by these primers are still in the linear range (L. J. Windsor,

unpublished data). The cyclophilin band was set as the baseline and was then compared to each MMP band to obtain the relative expression level of each MMP or TIMP. The relative expression levels between the *P. gingivalis* treated and untreated cells were then determined.

Results

Collagen degradation

P. gingivalis supernatant at dilutions greater than 5% v/v was able to increase the collagen degrading ability of the HGFs, which was evident by the fact that all the collagen in the wells was cleaved by day 4 (data not shown). In the 6-day collagen degradation assays, progressive dissolution of collagen was observed only underneath the cell colonies in the wells with untreated HGFs (Fig. 1A). In the wells with 10% *P. gingivalis* supernatant treated HGFs, all the collagen in the well was cleaved by day 4 (Fig. 1A). When the *P. gingivalis* supernatant was denatured by heat, there was no increase in collagen degradation and the amount of collagen degradation observed was similar to that mediated by the untreated HGFs (Fig. 1B). No collagen degradation could be observed with 10% *P. gingivalis* supernatant alone (Fig. 1B).

Gelatin zymography

Gelatinase-A (72/67 kDa) bands were detected in the *P. gingivalis* treated and untreated HGF conditioned media when the samples were loaded with non-reducing dye (Fig. 2). It was observed that with the addition of 1% and 10% *P. gingivalis* supernatant in the media, two proteolytic bands of approximately 57/52 kDa (latent MMP-1) weakened and two bands around 47/42 kDa (active MMP-1) emerged (Fig. 2). The 47/42 kDa bands were more prevalent in the conditioned media from the HGFs treated with 10% *P. gingivalis* supernatant than from the HGFs treated with 1% *P. gingivalis* supernatant, thus demonstrating that the effect was dose-dependent (Fig. 2).

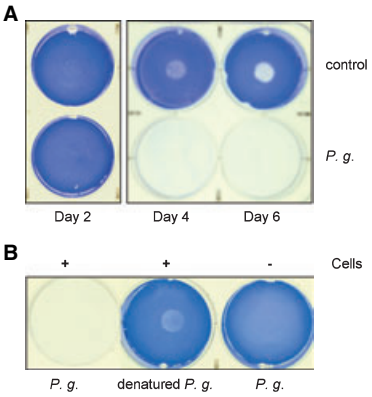

Fig. 1. Collagen degradation. Human gingival fibroblasts (HGFs) were seeded as single colonies (50,000 cells/well) in 6-well plates coated with reconstituted rat-tail Type I collagen (0.45 mg/well). After select time periods, HGFs were removed and the collagen cleavage visualized by Coomassie blue staining. (A) Cell-mediated collagen degradation at 2, 4, and 6 days. Control: serum-free DME media without *P. gingivalis* supernatant; *P. g.*: serum-free DME media with 10% v/v *P. gingivalis* supernatant. (B) Day 4 collagen degradation with/without HGFs. HGFs were cultured in serum-free DME media containing 10% v/v *P. gingivalis* supernatant (*P. g.*) or 10% v/v denatured *P. gingivalis* supernatant (denatured *P. g.*). Ten per cent v/v *P. gingivalis* supernatant without HGFs served as an additional control (– cells).

Western blot

Latent MMP-1 (57/52 kDa), MMP-2 (72 kDa), MMP-3 (60/54 kDa), and MMP-14 (64 kDa) were detected in both the treated and untreated HGF conditioned media (Fig. 3). Active MMP-1 (47/42 kDa), MMP-3 (44 kDa), and MMP-14 (54 kDa), as well as a partial active form of MMP-2 (67 kDa), were detected only in the *P. gingivalis* treated HGF conditioned media (Fig. 3). Proteolytic activation of the MMPs was determined by an approximately 10 kDa reduction in molecular weight. The amount of the TIMP-1 protein in the *P. gingivalis* treated HGF conditioned media decreased compared to that in the untreated HGF conditioned media (Fig. 4A), whereas the TIMP-2 protein level appeared unaltered (Fig. 4A).

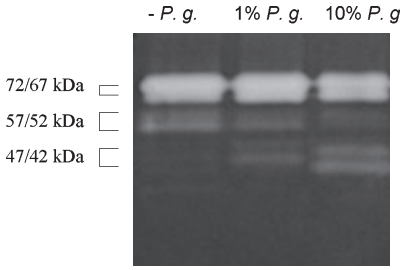

Fig. 2. Zymography. Human gingival fibroblast (HGF) conditioned media in non-reducing dye (without β-mercaptoethanol) was resolved in sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels containing 1 mg/ml gelatin. Lane 1: conditioned media from untreated HGFs; lane 2: conditioned media from HGFs treated with 1% v/v *P. gingivalis* supernatant; lane 3: conditioned media from HGFs treated with 10% v/v *P. gingivalis* supernatant.

Direct degradation of tissue inhibitors of matrix metalloproteinases by *Porphyromonas gingivalis* supernatant

Porphyromonas gingivalis supernatant could directly degrade purified TIMP-1. Figure 5A shows the western blot of the 2 h degradation assay for TIMP-1. The degradation of TIMP-1 could be inhibited by inclusion of the cysteine proteinase inhibitor E64 (Fig. 5A). However, TIMP-2 could not be degraded by the *P. gingivalis* supernatant (Fig. 5B), even when the assay was extended to 48 h (data not shown).

Reverse transcription–polymerase chain reaction

The mRNA expression levels of multiple MMPs and TIMPs from HGF cultures treated with or without 10% *P. gingivalis* supernatant for 48 h were determined. Both treated and untreated HGFs were shown to express mRNA for MMP-1, MMP-2, MMP-3, MMP-11, MMP-14, and MMP-15, as well as mRNA for TIMP-1 and TIMP-2 (Fig. 6). MMP-12 was expressed only by the *P. gingivalis* treated HGFs (Fig. 6). NIH image analyses revealed that MMP-1 mRNA expression from the treated

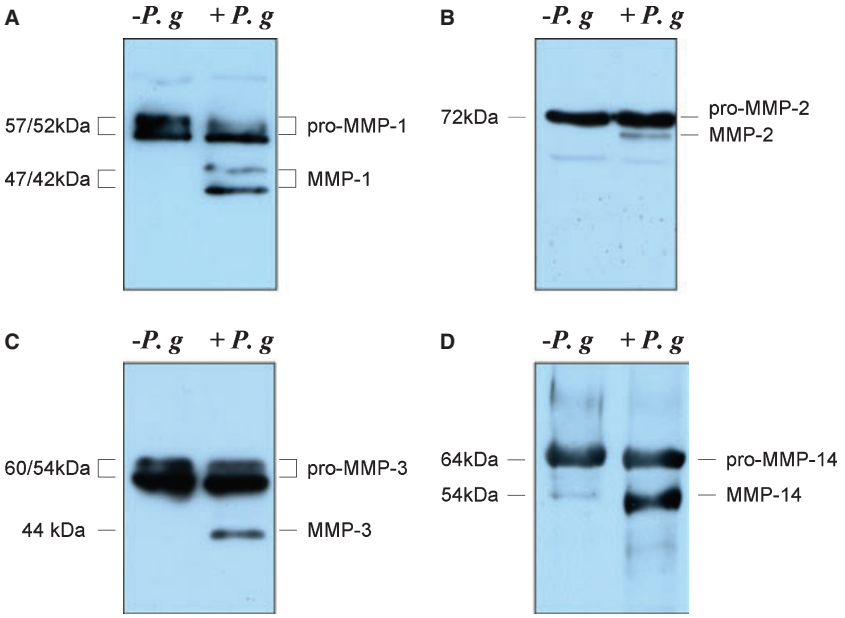

Fig. 3. Western blots of the matrix metalloproteinases (MMPs) in human gingival fibroblast (HGF) conditioned media. (A) MMP-1 (collagenase-1). (B) MMP-2 (gelatinase-A). (C) MMP-3 (stromelysin-1). (D) MMP-14 (MT1-MMP). *–P. g.*: conditioned media from untreated HGFs; *+P. g.*: conditioned media from HGFs treated with 10% v/v *P. gingivalis* supernatant.

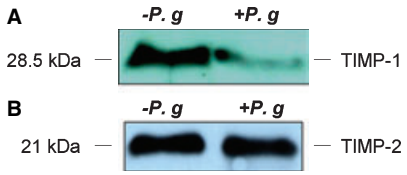

Fig. 4. Western blots of tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) and TIMP-2 in human gingival fibroblast (HGF) conditioned media. (A) TIMP-1. (B) TIMP-2. *–P. g.*: conditioned media from untreated HGFs; *+P. g.*: conditioned media from HGFs treated with 10% v/v *P. gingivalis* supernatant.

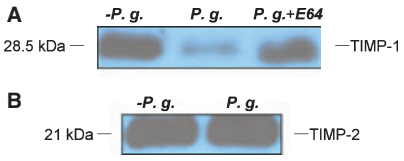

Fig. 5. Western blots of purified tissue inhibitors of matrix metalloproteinases (TIMPs) after 2 h treatment with *P. gingivalis* supernatant. (A) TIMP-1. (B) TIMP-2. TIMP-1 was incubated without *P. gingivalis* supernatant (*–P. g.*), with *P. gingivalis* supernatant (*P. g.*), and with *P. gingivalis* supernatant pre-incubated with 0.1 mM E64 (*P. g.* + E64). TIMP-2 was incubated with/without *P. gingivalis* supernatant.

HGF culture increased 2.14-fold over the untreated HGFs (Table 1). The MMP-3 mRNA level was unchanged. MMP-2 mRNA level was slightly increased in the presence of *P. gingivalis*. MMP-14 (membrane type-1 MMP) mRNA decreased, whereas MMP-15 (membrane type-2 MMP) increased in the presence of *P. gingivalis*. The mRNA expression of TIMP-1 and TIMP-2 by the treated HGF also increased (1.58 and 1.68-fold compared to the untreated control, respectively).

Discussion

Gingival fibroblasts are by far the most common cells in the gingiva (27). They play a major role in maintaining homeostasis in the periodontium by producing the extracellular matrix and the MMPs that have the capacity to remodel the extracellular matrix. In the current study, the collagen degrading ability of human gingival fibroblasts in the presence of *P. gingivalis* supernatant was examined for the first time, although similar studies have been conducted with rat epithelial cells and human foreskin fibroblasts (22, 23). It was demonstrated that greater than 5% v/v *P. gingivalis* supernatant caused an increase in the collagen degrading ability of HGFs. Progressive dissolution of collagen fibrils by HGFs was observed in the 6-day experiments and the collagen degradation progressed more rapidly in the HGFs treated with *P. gingivalis* supernatant. Collagen degradation by the *P. gingivalis* supernatant treated HGFs was not localized to the area underneath the cell colonies, but occurred throughout the wells after 4 days of incubation. This collagen cleavage pattern was different from that mediated by epithelial cells with the treatment of 10% *P. gingivalis* supernatant in which the increased collagen degradation was localized to underneath the cell colonies (23). The increased collagen degradation in the current study was also demonstrated to be host-mediated because the *P. gingivalis* supernatant had little or no collagen degrading ability without the presence of the HGFs.

Crude *P. gingivalis* supernatant was utilized as the stimuli in this study because the focus was on the host effect and not on the active components of the *P. gingivalis* supernatant. Therefore, the *P. gingivalis* components that were responsible for the effect on the altered host collagen degrading ability were not determined. However, there was no increase in the collagen degradation observed in the wells where only boiled *P. gingivalis* supernatant was added. This indicated that the factor(s) in the bacterial supernatant was responsible for modulating the host-mediated collagen degradation and was capable of being denatured, thus suggesting that the effect was protein-mediated. Cysteine proteinases (gingipains) account for the major proteolytic activity of *P. gingivalis* and they play various roles in the development of periodontal disease, including the development of edema, neutrophil infiltration, and bleeding (15). Gingipains exhibit enzymatic activity against many host proteins such as immunoglobulins, extracellular matrix proteins,

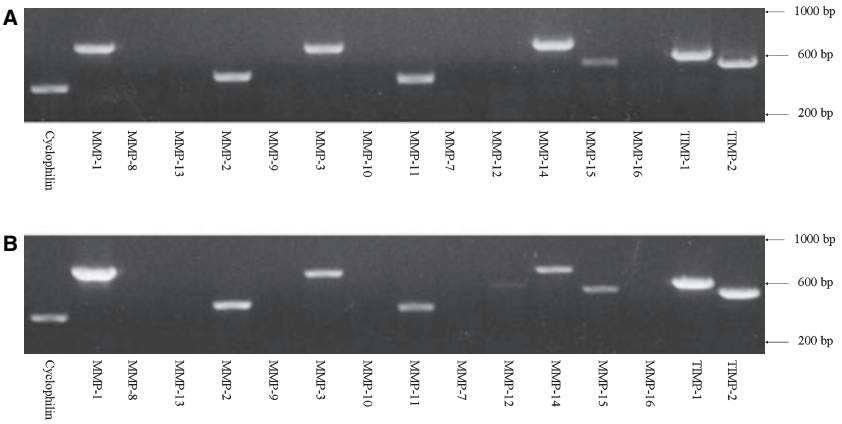

Fig. 6. Reverse transcription–polymerase chain reaction (RT–PCR) of untreated human gingival fibroblasts (HGFs) (A) and 10% v/v *P. gingivalis* treated HGFs (B). The RT–PCR products were resolved in 1% agarose gels after 30 PCR cycles and visualized with UV light after staining with ethidium bromide. MMP-1: collagenase-1; MMP-8: collagenase-2; MMP-13: collagenase-3; MMP-2: gelatinase-A; MMP-9: gelatinase-B; MMP-3: stromelysin-1; MMP-10: stromelysin-2; MMP-11: stromelysin-3; MMP-7: matrilysin; MMP-12: macrophage MMP; MMP-14: membrane-type 1 MMP; MMP-15: membrane-type 2 MMP; and MMP-16: membrane-type 3 MMP. MMP: matrix metalloproteinase; TIMP: tissue inhibitor of MMP.

Table 1. Comparison of reverse transcription–polymerase chain reaction products from treated human gingival fibroblasts vs. untreated human gingival fibroblasts

	Treated /Untreated*
Cyclophilin	1.00 ± 0.00
MMP-1 (collagenase-1)	2.14 ± 0.017
MMP-8 (collagenase-2)	ND
MMP-13 (collagenase-3)	ND
MMP-2 (gelatinase-A)	1.25 ± 0.103
MMP-9 (gelatinase-B)	ND
MMP-3 (stromelysin-1)	1.01 ± 0.013
MMP-10 (stromelysin-2)	ND
MMP-11 (stromelysin-3)	0.83 ± 0.026
MMP-7 (matrilysin)	ND
MMP-12 (macrophage MMP)	ND: untreated D: treated
MMP-14 (MT1-MMP)	0.67 ± 0.018
MMP-15 (MT2-MMP)	1.41 ± 0.029
MMP-16 (MT3-MMP)	ND
TIMP-1	1.58 ± 0.029
TIMP-2	1.68 ± 0.040

ND: not detected; D: detected; MMP: matrix metalloproteinase; TIMP: tissue inhibitor of MMPs.
*Average ± standard deviation ($n = 3$).

bactericidal proteins and peptides, iron transporting factors and inhibitors, and proteins involved in the coagulation, complement, and kallikrein-kinin cascades, as well as host proteinases and their inhibitors (28, 29). Previously, it has been demonstrated that a purified extracellular thiol-proteinase from *P. gingivalis* ATCC 33277 could stimulate epithelial cells to degrade a collagen Type I fibril matrix (23) and that this proteinase was homogenous to Lys-gingipain and could not directly degrade collagen fibrils (30). Therefore, it is tempting to speculate that the gingipains released from *P. gingivalis* are responsible for the altered collagen degrading ability of the HGFs demonstrated in this study. Future studies are required to determine the role of the gingipains and other *P. gingivalis* factors in the modulation of host-mediated collagen degradation.

Altered MMP activities from the host cells were responsible, in part, for the increased collagen degradation induced by *P. gingivalis* supernatant. Zymography analyses in the current study revealed that two proteolytic bands of approximately 57/52 kDa (latent MMP-1) weakened and two bands around 47/42 kDa (active MMP-1) emerged in the conditioned media with *P. gingivalis* supernatant treatment. This demonstrated that activation had occurred. Western blot results demonstrated that MMP-1, MMP-2, MMP-3, and MMP-14 had more readily undergone activation in the HGFs conditioned media with *P. gingivalis* supernatant. This is the first time that an increase in the activation of MMP-14 has been demonstrated. The 64 kDa proenzyme of MT1-MMP was detected in both the treated and untreated HGF cell media, whereas the 54 kDa active form of MT1-MMP was only detected in the treated HGF conditioned media. The activation of MMP-1, MMP-2, and MMP-3 confirmed previous reports that these MMPs could be activated by *P. gingivalis* supernatant and gingipains (16–18,23). MMP-9 has been shown to be the main gelatinase in oral fluids of healthy subjects and periodontitis patients (7) and it has been reported to be expressed by human gingival epithelial cells (20) and human periodontal ligament fibroblasts (17). However, no MMP-9 expression by these HGFs could be detected by RT–PCR, zymography, or western blot analyses in this study (data not shown).

Porphyromonas gingivalis supernatant also altered the balance of the MMPs to the TIMPs by modulating the TIMP levels. In the current study, the protein and mRNA levels of TIMP-1 and TIMP-2 from HGFs in the presence of *P. gingivalis* supernatant were compared for the first time. The direct digestion of both purified TIMP-1 and TIMP-2 by *P. gingivalis* supernatant was also examined for the first time. It was shown that with no decrease in mRNA level, the TIMP-1 protein level in the conditioned media of HGFs treated with 10% *P. gingivalis* supernatant was decreased compared to the untreated control. The lower TIMP-1 protein level in the treated conditioned media observed might then be due, in part, to TIMP-1 degradation by the secreted cysteine proteinases in the *P. gingivalis* supernatant. In support of this, the addition of the cysteine proteinase inhibitor blocked the digestion of TIMP-1 by the *P. gingivalis* supernatant. It has been reported that planktonic cells or biofilms of *P. gingivalis* could degrade purified TIMP-1 into several lower molecular mass fragments (31). In another study, the TIMP-1 protein produced by dental pulp cells was

slightly elevated by *P. gingivalis*, whereas the TIMP-2 protein level was inhibited (21). The TIMP-2 level in the HGFs conditioned media was not altered by the addition of the *P. gingivalis* supernatant. Also, the purified TIMP-2 protein could not be digested by the *P. gingivalis* supernatant. The assay results for the degradation of purified TIMP-1 and TIMP-2 might explain the different effects that *P. gingivalis* supernatant had on TIMP-1 and TIMP-2 levels in the HGF conditioned media.

It has been reported by DeCarlo *et al.* (23) that purified *P. gingivalis* proteinases could up-regulate the mRNA level of collagenase-1 (MMP-1) and stromelysin-1 (MMP-3) in epithelial cells. Pattamapun *et al.* (18) also reported the up-regulation of MT1-MMP (MMP-14) mRNA in human periodontal ligament cells by *P. gingivalis* supernatant. For the first time, mRNA expression profiles of 13 MMPs and two TIMPs from HGFs were analyzed by RT–PCR. Expression of the mRNA for MMP-1, MMP-2, MMP-3, MMP-11, MMP-14, and MMP-15, as well as TIMP-1 and TIMP-2, were detected in both treated and untreated HGFs. No MMP-9 mRNA expression could be detected in either the treated or untreated HGFs. MMP-1 mRNA expression from HGFs treated with *P. gingivalis* supernatant increased 2.14-fold over the untreated HGFs. This finding may help to explain the increased collagen degrading ability of the HGFs. MMP-2 mRNA had a slight increase with *P. gingivalis* treatment. MMP-3 mRNA was unchanged, although more active MMP-3 protein was detected in the treated HGF conditioned media. MMP-12 mRNA was induced by *P. gingivalis* treatment and the significance of this induction is unclear. MMP-14 (MT1-MMP) mRNA decreased with *P. gingivalis* treatment, whereas MMP-15 (MT2-MMP) mRNA increased. The mRNA expression of TIMP-1 and TIMP-2 by the treated HGF was also increased, although western blot analyses showed that the TIMP-1 protein level decreased while the TIMP-2 protein level was unaltered. This demonstrated that the regulation of the TIMPs by *P. gingivalis* is complicated and occurred at multiple levels. As it is discussed in the model proposed by Strongin *et al.* (32), MT1-MMP and TIMP-2 are involved in MMP-2 activation. In this model, latent pro-MMP-2 binds to the MT1-MMP by using TIMP-2 as a bridging molecule to form a trimolecular complex. Subsequently, another MT1-MMP molecule approaches the complex and activates MMP-2 by cleaving its propeptide domain. Therefore, some MT1-MMP and TIMP-2 are associated with the cell membrane and can not be detected in the conditioned media. This might help explain why with the treatment of *P. gingivalis* supernatant, the MT1-MMP mRNA and TIMP-2 mRNA levels did not match the protein levels. The changes in mRNA of the MMPs and TIMPs are considered relatively small. However, the rate of collagen degradation was drastically increased. It is believed that this is due largely to the ability of *P. gingivalis* to increase the rate of MMP activation in this collagen degradation system. It is the lack of MMP activation that is the limiting step in collagenolysis in this system. It is a well-known fact that cells can secrete latent MMPs in large quantities, but if there is no activation of these MMPs then there can be no extracellular matrix degradation.

This is the first study in which the collagen degrading ability of human gingival fibroblasts in the presence of *P. gingivalis* supernatant has been examined. It is also the first time that the activation of MMP-14 has been observed to occur with the addition of *P. gingivalis* supernatant to HGFs and the first time that the mRNA profiles of 13 MMPs from HGFs have been examined in the presence of *P. gingivalis* supernatant. The mRNA levels of both TIMP-1 and TIMP-2 from HGFs in the presence of *P. gingivalis* supernatant, as well as the levels of purified TIMPs after the direct digestion by *P. gingivalis* supernatant, have also been compared for the first time.

In summary, it was demonstrated in this study that *P. gingivalis* increased the collagen degrading ability of HGFs, in part, by increasing MMP activation and by lowering the TIMP-1 protein level, as well as by affecting the mRNA expression of multiple MMPs and TIMPs.

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