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# Heterogeneity in the collagen-degrading ability of *Porphyromonas gingivalis*-stimulated human gingival fibroblasts

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*Background and Objective:* The purpose of this study was to characterize the heterogeneity of the collagen-degrading ability of different human gingival fibroblast cell lines treated with *Porphyromonas gingivalis* supernatant.

*Material and Methods:* Seven human gingival fibroblast cell lines were analyzed for their ability to cleave Type I collagen in the presence and absence of culture supernatant from *P. gingivalis* ATCC 33277 (10% v/v). The matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) produced by these human gingival fibroblasts were monitored at the protein level by zymography and/or western blot analyses, as well as at the mRNA level by reverse transcription–polymerase chain reaction.

*Results:* The collagen-degrading ability of the human gingival fibroblasts increased in four cell lines (aggressive) and was only slightly altered in the other three cell lines (nonaggressive) in the presence of *P. gingivalis* supernatant. MMP-1, MMP-2, and MMP-3 more readily underwent activation while the TIMP-1 level was decreased in the conditioned media from a *P. gingivalis*-treated human gingival fibroblast aggressive cell line. None of these was altered in a nonaggressive cell line. The mRNA levels of the MMPs and TIMPs were only slightly different between these two cell lines.

*Conclusion:* Heterogeneity exists in human gingival fibroblasts in regard to their collagenolytic activity in the presence of *P. gingivalis*.

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Periodontal disease has been recognized to be a host-mediated inflammatory response to the pathogenic subgingival microflora. However, individuals with the same periodontal pathogens may experience different disease processes and/or severity (1). Susceptibilities to periodontal disease have been demonstrated to be associated with certain genotypic and phenotypic characteristics (1). For example, interleukin-1 gene polymorphisms have been shown to influence the onset of periodontal disease and treatment responses (2,3). In addition, fibroblast subtypes with phenotypic heterogeneities have been associated with drug-induced gingival overgrowth (4).

Gingival fibroblasts are the predominant cell type in periodontal connective tissues. These cells produce components of the extracellular matrix, as well as the enzymes that degrade the extracellular matrix (5). *In vitro* studies have shown that there are differences in cell size and shape (6), proliferative rate (6,7), collagen

production (8–10), and expression of growth factor receptors (11), between fibroblasts obtained from healthy and from inflamed periodontal tissues.

Although periodontal disease is thought to be initiated by a number of gram-negative anaerobic bacteria, the tissue destruction that occurs in periodontal disease is believed to be the result of host responses to these bacteria and their products. *Porphyromonas gingivalis* (*P. gingivalis*) is a gram-negative, anaerobic, nonmotile, nonsporulating coccobacillus (12). *In vitro* studies have demonstrated that *P. gingivalis* and its products can affect host cell-mediated collagen degradation and the levels of the matrix metalloproteinases (MMPs) produced by these host cells (13–24). The MMPs are a family of zinc-dependent endopeptidases that contain at least 28 members; these are divided into several subfamilies, which include the collagenases, gelatinases, stromelysins, membrane-type MMPs, minimal-domain MMPs, and others. The MMPs produced by host cells (e.g. neutrophils, macrophages, fibroblasts, osteoblasts, and osteoclasts), together with other neutral proteinases, such as neutrophil elastase, are responsible for the connective tissue breakdown that occurs in the gingival and periodontal tissues during periodontal disease (25). *P. gingivalis* can up-regulate MMP-1, MMP-3, and MMP-14, as well as promote their activation and the activation of MMP-2, MMP-8, and MMP-9 (13–24). *P. gingivalis* has also been reported to affect the tissue inhibitors of matrix metalloproteinases (TIMPs), for example by directly degrading TIMP-1 protein (13,26).

To date, no study has been conducted comparing the collagen-degrading ability of different human gingival fibroblast cell lines when exposed to *P. gingivalis* stimulation. Therefore, the objectives of this study were to characterize the collagen-degrading ability of several human gingival fibroblast cell lines in the presence and absence of culture supernatant from *P. gingivalis*, as well as to compare the MMPs and TIMPs produced by these cell lines under the same culture conditions.

## Material and methods

### Human gingival fibroblast cell culture and bacterial culture supernatant

Seven human gingival fibroblast cell lines were established from tissue explants of clinically healthy gingival connective tissue obtained, with Institutional Review Board approval, from seven different patients undergoing crown-lengthening surgeries at Indiana University School of Dentistry. The tissue explants were washed twice in 70% ethanol and rinsed in phosphate-buffered saline (PBS) to remove the ethanol. The tissues were then minced into small fragments of $\approx 1\ mm^3$, placed in cell culture dishes, and air-dried. Subsequently, the tissues were cultured in low-glucose (1 g/L) Dulbecco's modified Eagle's (DME) medium (Hyclone, Logan, UT, USA), supplemented with 15% fetal bovine serum (Hyclone), 4 mM L-glutamine (Hyclone), 100 U/mL penicillin, 50 μg/mL gentamicin, and 2.5 μg/mL fungizone (Invitrogen, Carlsbad, CA, USA) for 5–7 d at 37°C and 5% $CO_2$. The cells that grew out of the explants were subcultured and maintained. Cells of passages 3–8 were utilized in the experiments.

The *P. gingivalis* ATCC 33277 supernatant was obtained as a generous gift from Dr J. Katz (University of Alabama at Birmingham School of Dentistry, Birmingham, AL, USA). *P. gingivalis* was cultured in supplemented brain heart infusion growth media, as previously described (13). The collected supernatant was filtered twice through 0.2-μm membranes and then stored at −20°C.

### Cell-mediated collagen-degradation assay

The collagen-degrading ability of these seven human gingival fibroblast cell lines was determined with a reconstituted Type I collagen assay system, as previously described (13). Briefly, a thin layer of rat tail tendon Type I collagen was coated onto six-well plates (450 μg of collagen per well). Human gingival fibroblasts at 90% confluence were collected and seeded as single colonies (50,000 cells/well) in the center of the collagen-coated six-well plates. After the cells attached, 2 mL of serum-free DME medium containing 10% (v/v) *P. gingivalis* supernatant was added to each well. Serum-free medium, medium containing 10% bacteria growth medium, and medium containing 10% denatured *P. gingivalis* supernatant (boiled at 100°C for 15 min), were utilized as experimental controls. A synthetic MMP inhibitor (GM6001, 100 nM; Chemicon, Temecla, CA, USA) and a cysteine protease inhibitor [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane, E64, 0.1 mM; Sigma Chemical Co., St Louis, MO, USA] were utilized in inhibition experiments. After specific experimental periods (1–6 d), the conditioned media from the human gingival fibroblast cells were collected for zymography and/or western blot analyses. Collagen cleavage by the human gingival fibroblasts in each well was then visualized by staining the plates with Coomassie blue after removal of the cells with 500 μL of 0.1% Triton X-100 (Sigma Chemical Co.) containing 200 μL of 0.25% trypsin (Invitrogen). The experiments were repeated at least three times.

### Soluble collagen cleavage assay

Based on the results of the *P. gingivalis*-stimulated human gingival fibroblast-mediated collagen-degradation assay, one human gingival fibroblast cell line was chosen from the cell lines whose collagen-degrading ability was increased (aggressive cell lines) and one from the cell lines whose collagen-degrading ability was unaltered/decreased (nonaggressive cell lines), for further analyses. Conditioned serum-free culture media from these two cell lines were collected on day 4. The following samples (90% v/v) – Tris buffer, untreated human gingival fibroblast conditioned media, and conditioned media from human gingival fibroblasts treated with 10% *P. gingivalis* supernatant – were incubated separately with 250 μg/mL Type I collagen in

0.5 M glucose and Tris buffer (50 mM Tris, pH 7.4, 0.2 M NaCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, and 15 mM $NaN_3$), for 24 h at 37°C. Additional samples of *P. gingivalis*-untreated human gingival fibroblast conditioned media from the aggressive and nonaggressive cell lines were preincubated with 10% *P. gingivalis* supernatant for 24 h before being incubated with collagen to determine if the *P. gingivalis*-enhanced collagen cleavage required the cells to be present. The incubated samples were then mixed with 6× loading dye containing 2-mercaptoethanol and resolved at 200 V by 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The gels were then stained with Coomassie blue to visualize the collagen bands. The experiments were repeated at least three times.

### Gelatin zymography

The human gingival fibroblast-conditioned media from the seven cell lines were collected on day 4 and their protein concentrations determined according to the Bio-Rad Protein Assay protocol (Bio-Rad Laboratories, Hercules, CA, USA). Media with equal amounts of total protein were then mixed with nonreducing loading buffer (without 2-mercaptoethanol) and resolved at 200 V in 10% 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, before the gels were incubated in fresh solution 3 overnight at 37°C. The gels were stained with Coomassie blue and then destained to visualize the proteolytic bands.

### Western blot analyses

To monitor the protein levels of select MMPs and TIMPs produced by the aggressive cell line and the nonaggressive cell line, 48-h human gingival fibroblast culture media from each cell line were collected and concentrated 16-fold with Centriprep centrifugal filters (Millipore, Bedford, MA, USA). The protein concentrations in the samples were determined according to the Bio-Rad Protein Assay protocol (Bio-Rad Laboratories) before samples with equal amounts of total protein were mixed with reducing loading dye, boiled at 100°C for 10 min, and then resolved by SDS–PAGE (10% gels) at 200 V. The proteins on the gels were transferred to nitrocellulose membranes at 0.3 A for 1 h in blotting buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, and 10% methanol), after which the membranes were blocked for 1 h with 5% milk in PBS containing 0.1% Tween-20 (PBS-T, pH 7.4) and then incubated overnight, at 4°C, with the following primary antibodies: monoclonal Ab-5 (5 μg/mL, Clone III12b) for MMP-1; monoclonal Ab-2 (5 μg/mL, Clone VB3) for MMP-2; monoclonal Ab-2 (5 μg/mL, Clone IID4) for MMP-3; monoclonal Ab-2 (5 μg/mL, Clone IC3); for TIMP-2; polyclonal Ab 815 (0.3 μg/mL) for MMP-14; and polyclonal Ab 2315 (5 μg/mL) for TIMP-1. These primary antibodies were purchased from NeoMarkers (Fremont, CA, USA) except for the antibody to MMP-14 (Chemicon) and the antibody for TIMP-1 (a gift from Dr K. Bodden, Mobile, AL, USA). After washing three times with PBS-T, the membranes were incubated with antimouse or antirabbit secondary antibodies (Amersham, Piscastaway, NJ, USA) for 1 h at room temperature. An ECL™ kit (Amersham) was utilized to develop the membranes according to the manufacturer's protocol.

### Reverse transcription–polymerase chain reaction (RT–PCR)

Total RNA from human gingival fibroblasts (cultured for 48 h with or without *P. gingivalis* supernatant) from both the aggressive and the nonaggressive cell lines was extracted according to the protocol of the Qiagen RNeasy mini kit (Qiagen, Valencia, CA, USA). The PCR primers utilized for cyclophilin, 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), MMP-16 (membrane-type 3 MMP), TIMP-1, and TIMP-2, have been described previously (27). The mRNA and primers were mixed with the Qiagen OneStep RT–PCR mix (Qiagen), according to the manufacturer's protocol. RT–PCR was performed using a Bio-Rad iCycler (Bio-Rad Laboratories). The reverse transcription was accomplished at 50°C for 30 min and terminated by incubation at 95°C for 15 min. The 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 and stained with ethidium bromide. The gels were photographed under ultraviolet light and the photographs were then scanned and analyzed by NIH SCION IMAGE software (version beta 4.03). The cyclophilin bands were utilized to standardize the relative expression levels for each MMP and TIMP. The relative expression levels of the MMPs and TIMPs from the treated and untreated cells were then calculated and expressed as ratios.

## Results

### Cell-mediated collagen degradation

Untreated human gingival fibroblasts from all seven cell lines cleaved the collagen underneath the cell colonies progressively in the 6-d experimental period (data not shown). There was no passage-dependent variation observed with the human gingival fibroblasts from passages 3–8 in the cell-mediated collagen-degradation assays. In the presence of 10% *P. gingivalis* supernatant, four of the seven human gingival fibroblast cell lines cleaved all the collagen in the well by day 4 (Fig. 1). The collagen cleavage mediated by the human gingival fibroblasts in the other three cell lines treated with *P. gingivalis*

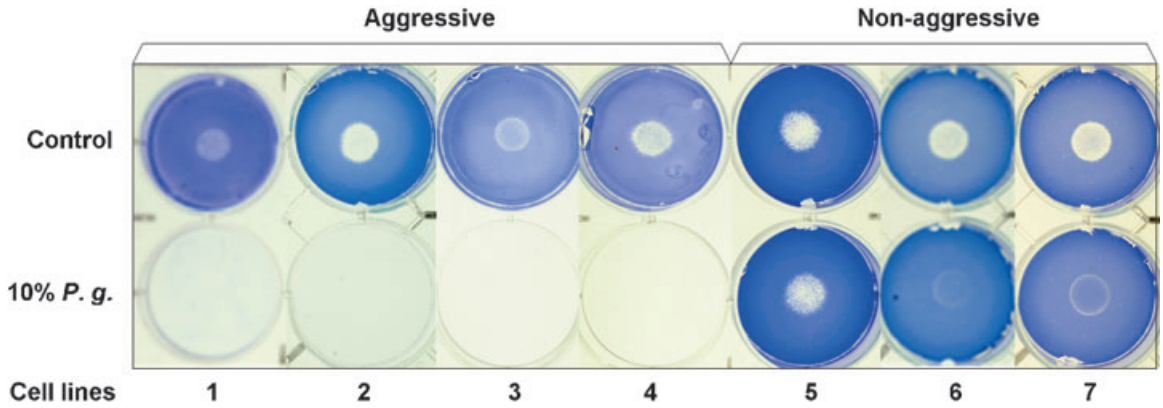


*Fig. 1.* Cell-mediated collagen-degradation assay. Human gingival fibroblasts were seeded as single colonies (50,000 cells/well) in the center of six-well plates coated with reconstituted rat-tail Type I collagen (0.45 mg/well). Control, serum-free Dulbecco's modified Eagle's (DME) medium was added; 10% *P. g.*, serum-free DME media containing 10% (v/v) of *Porphyromonas gingivalis* ATCC 33277 supernatant. On day 4, the human gingival fibroblasts were removed with Triton X-100 and trypsin. The collagen cleavage was then visualized by Coomassie blue staining.

supernatant was limited to the area underneath the cell colonies (Fig. 1). The level of collagen cleavage mediated by these three cell lines treated with *P. gingivalis* supernatant was equal to or less than that observed in the untreated controls (Fig. 1), even when the experiments were extended up to 8 d (data not shown). These results were reproducible for all of the cell lines in multiple experiments. One cell line was chosen from each of these two different groups for further analyses. The cell line that was more aggressive in degrading the collagen in the presence of *P. gingivalis* supernatant was referred to as the aggressive cell line. The cell line in which the collagen degradation was not enhanced when treated with *P. gingivalis* supernatant was referred to as the nonaggressive cell line.

The MMP inhibitor, GM6001, completely blocked the collagen cleavage observed in both the aggressive and the nonaggressive cell lines (data not shown). The cysteine protease inhibitor, E64, inhibited the increased collagen degradation observed in the aggressive cell line in the presence of *P. gingivalis* supernatant and had no effect on the collagen degradation observed in the *P. gingivalis* supernatant-treated nonaggressive cell line (data not shown).

### Collagen cleavage assay

*P. gingivalis* untreated culture media from both cell lines cleaved little to no soluble Type I collagen (Fig. 2, Media). Media from the aggressive cell line cultured in the presence of 10% *P. gingivalis* supernatant cleaved the Type I collagen (Fig. 2, Aggressive, + *P. gingivalis* media). There was little to no collagen cleavage mediated by the media from the nonaggressive cell line treated with the *P. gingivalis* supernatant (Fig. 2, Nonaggressive, + *P. gingivalis* media). Untreated human gingival fibroblast culture media from both cell lines preincubated with 10% *P. gingivalis* supernatant for 24 h cleaved little to no collagen (Fig. 2, Aggressive and Non-aggressive, Media + *P. gingivalis*).

### Gelatin zymography

Pro-MMP-2 (72 kDa) was detected in the conditioned media from the *P. gingivalis*-treated and -untreated human gingival fibroblasts in both cell lines (Fig. 3). A partially active form of

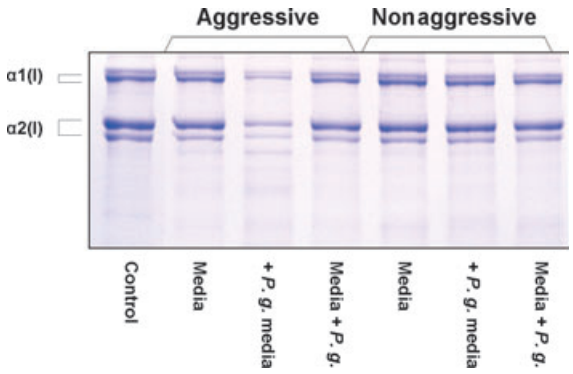


*Fig. 2.* Collagen cleavage in solution. Conditioned media were collected from human gingival fibroblast cell-mediated collagen-degradation plates (4-d culture). Samples (90% of total volume) were incubated with 250 μg/mL of Type I collagen and 0.5 M glucose in Tris buffer (pH 7.4). Control, collagen and Tris buffer; Media, collagen and untreated human gingival fibroblast-conditioned media; + *P. g.* media, collagen and conditioned media from human gingival fibroblasts treated with 10% *Porphyromonas gingivalis* supernatant; Media + *P. g.*, collagen and untreated human gingival fibroblast-conditioned media preincubated with 10% *P. gingivalis* supernatant for 24 h. The incubated samples were then collected, mixed with 6× loading dye, and resolved at 200 V in 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels. The gels were then stained with Coomassie blue to visualize the collagen bands.

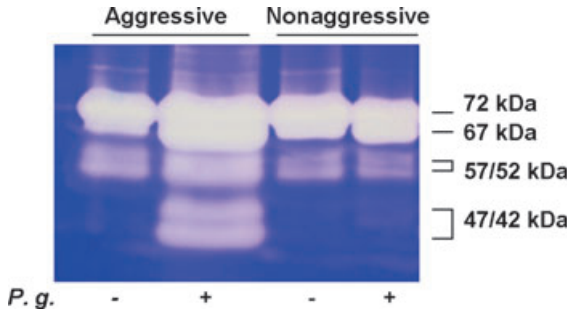


*Fig. 3.* Zymography. Conditioned media were collected from cell-mediated collagen-degradation plates (4-d culture) of the aggressive and the nonaggressive cell lines. Collected human gingival fibroblast-conditioned media samples in nonreducing dye (without 2-mercaptoethanol) were then resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) in gels containing 1 mg/mL gelatin. –*P. g.*, conditioned human gingival fibroblast culture media; + *P. g.*, conditioned media from human gingival fibroblasts cultured with 10% *Porphyromonas gingivalis* supernatant.

MMP-2 (67 kDa) was more prevalent in the media from the *P. gingivalis* supernatant-treated aggressive cell line (Fig. 3). Proteolytic bands of 57/52 kDa were observed in both the aggressive and the nonaggressive cell lines (Fig. 3). Two proteolytic bands of ≈47/42 kDa emerged in the media from the aggressive cell line in the presence of 10% *P. gingivalis* supernatant (Fig. 3).

### Western blot

Pro-MMP-1 (57/52 kDa), pro-MMP-2 (72 kDa), pro-MMP-3 (60/54 kDa), and pro-MMP-14 (64 kDa) were all detected in the conditioned media from both cell lines regardless of being treated or untreated with *P. gingivalis* supernatant (Fig. 4). The active forms of MMP-1 (47/42 kDa) and MMP-3 (50/44 kDa) were only observed in the conditioned media from the aggressive cell line treated with *P. gingivalis* supernatant (Fig. 4). However, the active form of MMP-14 (membrane type-1 MMP, 54 kDa) was detected in the conditioned media from both cell lines when treated with the *P. gingivalis* supernatant. The amount of the TIMP-1 protein in the media of the *P. gingivalis*-treated aggressive cell line was lower than that in the untreated sample (Fig. 4). The TIMP-2 protein level was unaltered in both cell lines in the presence or absence of the *P. gingivalis* supernatant (Fig. 4). Less MMP-1, MMP-3, and TIMP-2 were expressed in the nonaggressive cell line (Fig. 4).

### RT–PCR

The mRNA levels of multiple MMPs and TIMPs from the nonaggressive cell line were compared with those from the aggressive cell line from a previous study by Zhou & Windsor (13). In both cell lines, mRNAs for cyclophilin, MMP-1, MMP-2, MMP-3, MMP-11, MMP-14, MMP-15, TIMP-1, and TIMP-2 were detected (Table 1). MMP-1 mRNA expression increased 2.14-fold in the *P. gingivalis*-treated aggressive cell line compared with the untreated control. The MMP-2 and

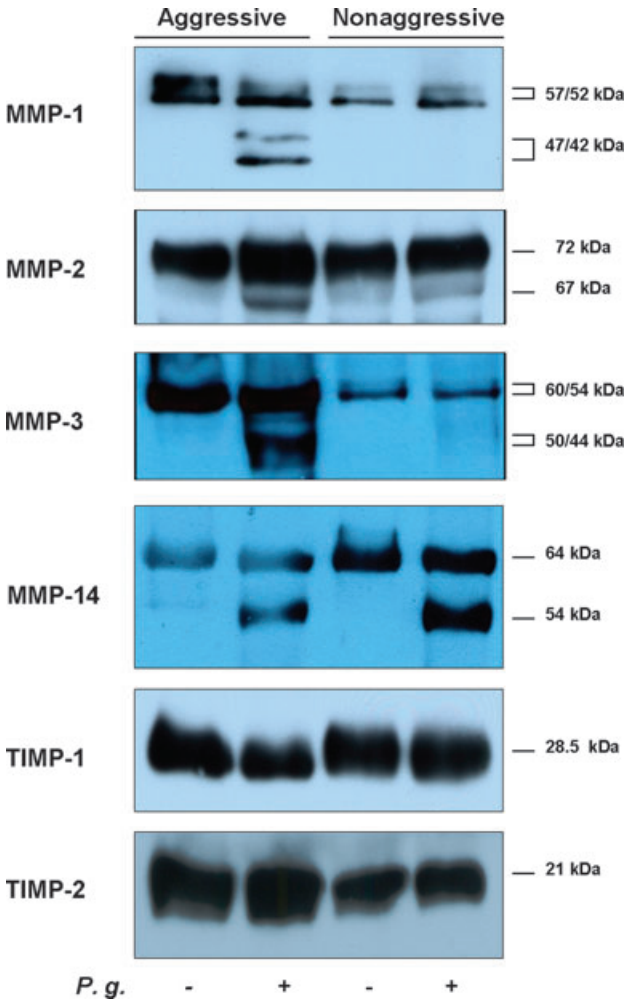


*Fig. 4.* Western blots of matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) in the conditioned media from the aggressive and the nonaggressive cell lines. –*P. g.*, conditioned media from untreated human gingival fibroblasts; + *P. g.*, conditioned media from human gingival fibroblasts cultured with 10% v/v *Porphyromonas gingivalis* supernatant.

*Table 1.* Reverse transcription–polymerase chain reaction (RT–PCR) results of the RNA extracted from the aggressive human gingival fibroblast cell line and the nonaggressive cell line treated with and without *Porphyromonas gingivalis* supernatant. The amount of RT-PCR products from the treated versus the untreated was expressed as a ratio (treated/untreated)

| | Aggressive cell line* treated/untreated† | Nonaggressive cell line treated/untreated† |
|---|---|---|
| Cyclophilin | 1.00 | 1.00 |
| MMP-1 (Collagenase-1) | 2.14 ± 0.017 | 0.78 ± 0.016 |
| MMP-8 (Collagenase-2) | ND | ND |
| MMP-13 (Collagenase-3) | ND | ND |
| MMP-2 (Gelatinase-A) | 1.25 ± 0.103 | 0.48 ± 0.026 |
| MMP-9 (Gelatinase-B) | ND | ND |
| MMP-3 (Stromelysin-1) | 1.01 ± 0.013 | 0.48 ± 0.015 |
| MMP-10 (Stromelysin-2) | ND | ND |
| MMP-11 (Stromelysin-3) | 0.83 ± 0.026 | 0.42 ± 0.010 |
| MMP-7 (Matrilysin) | ND | ND |
| MMP-12 (Macrophage MMP) | Induced/ND | 0.29 ± 0.007 |
| MMP-14 (membrane-type MMP-1) | 0.67 ± 0.018 | 0.78 ± 0.018 |
| MMP-15 (membrane-type MMP-2) | 1.41 ± 0.029 | 0.07 ± 0.002 |
| MMP-16 (membrane-type MMP-3) | ND | ND |
| TIMP-1 | 1.58 ± 0.029 | 0.74 ± 0.009 |
| TIMP-2 | 1.68 ± 0.040 | 0.67 ± 0.009 |

*Data for aggressive cell line from Zhou & Windsor (13).
†Average ± standard deviation ($n = 3$).
MMP, matrix metalloproteinase; ND, not detected; TIMP, tissue inhibitor of MMP.

MMP-3 mRNA levels were basically unaltered, with or without treatment. The MMP-15, TIMP-1, and TIMP-2 mRNA levels increased with *P. gingivalis* treatment (Table 1). MMP-12 was induced in the presence of *P. gingivalis* in the aggressive cell line, whereas it decreased with *P. gingivalis* treatment in the nonaggressive cell line (Table 1). MMP-1, MMP-2, MMP-3, TIMP-1, and TIMP-2 mRNAs decreased slightly in the *P. gingivalis*-treated nonaggressive cell line, whereas the MMP-15 mRNA decreased substantially (Table 1). MMP-14 and MMP-11 mRNAs slightly decreased in both cell lines with *P. gingivalis* treatment (Table 1).

## Discussion

Heterogeneity of fibroblasts in the periodontal tissues has been recognized in terms of anatomical location and with relevance to disease (28). Fibroblasts obtained from different anatomical locations and from diseased or healthy tissues can differ in their cell morphology and proliferative potential (6,7), alkaline phosphatase activity (29), response to growth factors and cytokines (11), collagen synthesis (8–10), cytoskeletal proteins and migratory behavior (30,31), and the production of matrix-degrading enzymes (32). In this study, it was revealed, for the first time, that human gingival fibroblast cell lines from different individuals demonstrate different responses to exogenous bacterial stimulation in regard to their collagen-degrading ability. Treatment with *P. gingivalis* supernatant increased the collagen-degrading ability of several cell lines (aggressive phenotypes). In contrast, the same treatment did not alter, or even decreased, the collagen-degrading ability of other cell lines (nonaggressive phenotypes).

MMPs were differentially regulated by the *P. gingivalis* supernatant in regard to their activation and expression levels in the aggressive and nonaggressive cell lines. The TIMP levels were also differentially regulated. In the aggressive cell line, MMP-1, MMP-2, and MMP-3 more readily underwent zymogen activation when the cells were treated with the *P. gingivalis* supernatant. The MMP-1 mRNA level increased two-fold when the aggressive cells were treated with the *P. gingivalis* supernatant. Also in the aggressive cell line, the TIMP-1 protein level decreased while its mRNA level was not altered substantially. This can be explained, in part, by the ability of the *P. gingivalis* supernatant to degrade the TIMP-1 protein directly (13,26). Changes in the MMP and TIMP-1 levels can explain, in part, the increased collagen-degrading ability of the aggressive cell line. In contrast, the protein levels of MMP-1, MMP-2, MMP-3, and TIMP-1 were not altered in the *P. gingivalis*-treated nonaggressive cell line, and the mRNA levels for multiple MMPs and TIMPs decreased. MMP-14 appeared to be regulated similarly in both cell lines and therefore was assumed to play no role in the differences in the collagen-degrading ability of these two cell lines. In addition, TIMP-2 was not altered in either cell line in the presence of *P. gingivalis* supernatant. This also supports the conclusion that MMP-14 does not play a role in this enhanced collagen cleavage, as the TIMP-2 level is important for the activation of MMP-2 because it serves as a bridging molecule for pro-MMP-2 to be activated by MMP-14 (33). It is unclear why MMP-15 mRNA decreased substantially in the nonaggressive cell line (0.07 ± 0.002) and increased in the aggressive cell line (1.41 ± 0.029) with *P. gingivalis* treatment compared with no treatment. The role of this membrane-associated MMP requires further investigation. Overall, the changes in the activation levels of the MMPs are believed to be more relevant than the changes in mRNA level to the increased collagen degradation in the aggressive cell line because only activated MMPs can cleave collagen. The imbalance of the activated MMPs and TIMP-1, rather than changes of the membrane-associated MMPs, seems to be essential in explaining, in part, the difference in the collagen cleavage mediated by these cell lines.

The abilities of the aggressive and the nonaggressive cell lines to dissolve soluble Type I collagen were determined in order to confirm the findings of the cell-mediated collagen-degradation assays. The conditioned medium from the *P. gingivalis* supernatant-treated aggressive cells dissolved more of the soluble Type I collagen within 24 h than did the conditioned medium

from the *P. gingivalis* supernatant-treated nonaggressive cells. The collagen that was cleaved by the conditioned medium from the *P. gingivalis* supernatant-treated aggressive cells generated the characteristic collagen fragments generated by MMP cleavage of the α1(I) and α2(I) chains. It was hypothesized in this study that the collagen was cleaved by MMPs which were activated by proteinases in the *P. gingivalis* supernatant. This hypothesis was supported by previous reports that *P. gingivalis* supernatant and its cysteine proteinases (gingipains) can activate MMP-1, MMP-2, MMP-3, MMP-8, and MMP-9, as well as catalyze the superactivation of MMP-1 by MMP-3 (15,16,18,19,21, 34). Untreated and treated cell-free media pre-incubated with *P. gingivalis* supernatant did not result in any collagen cleavage. The culture media collected from the *P. gingivalis* supernatant-treated nonaggressive cell line also cleaved little to no collagen. These findings suggest that the activation of MMPs by factors in the *P. gingivalis* supernatant require the presence of the cells and is regulated differentially in human gingival fibroblast cell lines.

One possible explanation for the difference seen in these cells in regard to collagen cleavage is that the environmental conditions provided by these two cell lines regulate the function of the gingipains differentially. A major mechanism that regulates the activity of the gingipains is the environmental reducing-oxidative (redox) condition. These enzymes can be inactivated in an environment that contains low levels of reducing agents (18). This inactivation can occur during experimental procedures, such as dialysis of the bacterial supernatant. The inactivation of the gingipains is reversible because the dialyzed *P. gingivalis* supernatant supplemented with reducing agents had similar cellular stimulatory activity to the nondialyzed *P. gingivalis* supernatant (18). L-cysteine is by far the most effective *in vitro* activator of the gingipains. It can act both as a reducing agent and as a stimulatory agent for amidolytic activity of the cysteine proteinases (35,36). In the experimental conditions of the current study, human gingival fibroblasts from these different individuals might differentially maintain the redox environment by producing different amounts of oxidants and/or antioxidants. It has been demonstrated clinically that the local antioxidant capacity of the periodontium varies among different individuals (37,38). Interestingly, it has been reported that gingival crevicular fluid collected from healthy individuals contains more reduced glutathione than that collected from individuals with chronic periodontal disease (38). Therefore, the actual *in vivo* pathological processes may be far more complicated than those revealed by *in vitro* experiments. Future studies are needed to test this hypothesis and whether any therapeutic interventions can be developed based on these findings.

MMP polymorphisms are not likely to have attributed to the differentially regulated collagen-degrading ability in these two cell lines because most MMP polymorphisms are single nuclear polymorphisms in the promoter regions of the MMPs (39–42) that influence only the gene expression of the MMPs. Also, it was concluded, from a previous study (13), that the activation of the MMPs, rather than their gene expression level, is more relevant to the enhanced collagen degradation seen in the aggressive cell line. Besides, previous studies have not demonstrated any association between the polymorphisms of the MMPs and periodontal disease (39–42).

This study demonstrated that heterogeneity exists in the collagen-degrading ability of human gingival fibroblasts. Crude *P. gingivalis* supernatant was utilized as the stimulus deliberately in order to mimic *in vivo* pathological conditions. Although it cannot be assumed that the *in vitro* findings reflect the *in vivo* situation, these results might help to explain, in part, the different susceptibilities and progression of periodontal disease seen in different individuals. This study also provides a knowledge base for future studies in determining the collagen-degrading ability of fibroblasts from different tissue locations or fibroblasts collected from diseased and healthy sites.

In summary, the results of this study showed that different human gingival fibroblast cell lines respond differentially to treatment with *P. gingivalis* supernatant in regard to their ability to degrade Type I collagen. The MMPs and TIMPs from the different cell lines were regulated differentially at the protein and/or mRNA levels. The imbalance of the activated MMPs and TIMP-1 seem to be essential in explaining the differences seen in the collagen cleavage mediated by these human gingival fibroblasts. It is probable that this heterogeneity plays a role in the variation seen in tissue remodeling associated with periodontal disease and that both genetic and environmental factors are involved in disease presentation and progression. The more that is known about the molecular and cellular mechanisms of the collagen-degradation process, the better equipped the field will be to provide new treatment options for periodontal disease.

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