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Effects of *Porphyromonas gingivalis* on human gingival fibroblasts from healthy and inflamed tissues

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Background and Objective: This study compared the ability of human gingival fibroblasts (HGFs) isolated from healthy and inflamed gingival tissues to degrade collagen in the presence and absence of *Porphyromonas gingivalis* supernatant.

Material and Methods: Human gingival fibroblasts were cultured from explants of 21 healthy and 21 inflamed periodontal tissues. The HGFs that grew out of the explants were seeded in the center of six-well plates coated with collagen in the presence and absence of 10% *P. gingivalis* supernatant. An inflamed and a healthy cell line were also evaluated with Arg-gingipain. After 6 days, Coomassie Blue was used to visualize the collagen cleavage.

Results: The collagen was totally cleaved in 12 (aggressive) of the 21 cell lines isolated from the inflamed tissues in the presence of *P. gingivalis*. The remaining nine cell lines (non-aggressive) cleaved only the collagen underneath the cell colonies in the presence of *P. gingivalis*. Of the healthy tissues, five (aggressive) of the 21 cell lines cleaved all the collagen and 16 cell lines (non-aggressive) only cleaved the collagen underneath the cell colonies in the presence of *P. gingivalis*. All the collagen was cleaved by an aggressive cell line and only the collagen underneath the cell colonies was cleaved by a non-aggressive cell line in the presence of Arg-gingipain.

Conclusion: The collagen in the wells was more readily cleaved by the inflamed than by the healthy cell lines, and the difference was statistically significant ($p = 0.0278$). Arg-gingipain gave identical results to the *P. gingivalis* supernatant.

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Periodontal disease is a complex process characterized by the destruction of the periodontal attachment apparatus following an inflammatory response (1). The initiation of the disease is multifactorial. The tissue destruction that occurs results from complex interactions between bacteria and the host (2).

Gingival fibroblasts are the predominant cell type in the periodontal connective tissue. These cells produce components of the extracellular matrix (ECM), as well as the enzymes that degrade the ECM (3). The degradation of the collagen-containing tissues at the dento-epithelial junction initiates the formation of a periodontal pocket (4). This can eventually result in inflammatory periodontal disease, which is characterized by a significant reduction in collagen fiber density in the gingival tissues, loss of Sharpey's fibers, and apical migration of the junctional epithelium onto the root surface (4).

The periodontopathogen *Porphyromonas gingivalis* has received significant attention because it is frequently isolated from periodontal pockets and because of its ability to produce multiple proteinases, which degrade a variety of substrates (5). In addition, *P. gingivalis* has been shown to affect

host-cell-mediated collagen degradation and the level of active matrix metalloproteinases (MMPs) produced by human gingival fibroblasts (HGFs; 5). The MMPs are a family of structurally related but genetically distinct enzymes that degrade ECM and basement membrane components (6). Activity of MMPs is controlled by changes in the delicate balance between the expression and activation of the MMPs and their major endogenous inhibitors, the tissue inhibitors of matrix metalloproteinases (TIMPs; 6). A body of evidence has demonstrated that the MMPs are expressed by a variety of cells derived from human periodontal tissues, which include polymorphonuclear cells, fibroblasts, macrophages and keratinocytes (7). The MMPs may be stimulated either directly by microbial products from the bacterial plaque that colonize the teeth and their surroundings or indirectly by inflammatory mediators generated in response to oral microorganisms (7). Release and activation of MMPs is affected by *P. gingivalis*, which can activate MMP-1, MMP-2, MMP-3, MMP-8 and MMP-9 (8,9).

Significant evidence exists to demonstrate that the MMPs play an important role in periodontal destruction (10). Numerous studies have demonstrated that tissue extracts and cultured tissue explants of inflamed human gingiva contain more collagenase activity than extracts and explants from healthy human gingiva (10). Collagenase activity in gingival crevicular fluid also increases during inflammation and has been correlated with the severity of the disease (10). Experimental gingivitis and periodontitis also display increased collagenase activity in diseased gingiva and the gingival crevicular fluid (10). All of these studies suggest that the MMP-mediated collagen-degrading ability of fibroblasts isolated from diseased tissues should exceed that of fibroblasts isolated from healthy tissues. Therefore, this study examined the ability of human gingival fibroblasts isolated from clinically healthy and inflamed gingival tissues to degrade collagen in the presence and absence of *P. gingivalis* supernatant.

Material and methods

Human gingival fibroblasts and bacterial culture supernatant

Human gingival fibroblasts were cultured from explants of 21 samples of clinically healthy gingival tissues from patients undergoing crown lengthening surgeries and 21 samples of inflamed gingival tissues (teeth exhibiting 6 mm probing depth or more) removed from patients undergoing periodontal surgery at Indiana University School of Dentistry with Institutional Review Board approval and patient written informed consent. The smoking status of the patients was noted. The tissues were transported from the clinic to the laboratory in phosphate-buffered saline (PBS) solution, washed with 70% ethanol, and rinsed in PBS to remove the ethanol. The washing and rinsing steps were repeated and then the tissues were minced into small fragments of approximately 1 mm^3. The tissue pieces were placed in cell culture dishes, air dried, and incubated for 5–7 days at 37°C and 5% CO_2 in low-glucose (100 mg/L) Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum (Hyclone), 200 mM L-glutamine, 100 U/mL penicillin, 50 μg/mL gentamycin and 0.25 μg/mL fungizone. The cells that grew out of the explants were subcultured and maintained. Cells at passage three to eight were used in the experiments.

Porphyromonas gingivalis ATCC 33277 supernatant was obtained as a generous gift from Dr Jannet Katz (University of Alabama at Birmingham School of Dentistry, Birmingham, AL, USA). The *P. gingivalis* were cultured in supplemented brain heart infusion growth medium (11). The collected supernatant was filtered twice through 0.2 μm membranes and then stored at −20°C until used.

Cell-mediated collagen degradation assay

The collagen-degrading ability of the HGFs was determined with a reconstituted collagen type I assay system described by Birkedal-Hansen (12). Briefly, rat tail tendon type I collagen was dissolved in 13 mM HCl and then mixed rapidly on ice with neutralizing phosphate buffer to give a final collagen concentration of 300 μg/mL. Aliquots of 1.5 mL per well (0.45 mg collagen) were dispensed in six-well plates and incubated at 37°C for 2 h. The collagen gels were then dehydrated overnight in a laminar flow hood, washed three times for 30 min with sterile water, and then air-dried in the hood. The HGFs were detached with 0.25% trypsin, pelleted, resuspended in serum-free DMEM, and then seeded as single colonies (50,000 cells per well) in the center of six-well plates coated with the collagen. After 6 days in serum-free DMEM with or without 10% *P. gingivalis* supernatant, the conditioned medium was removed. After the cells were removed with Triton X-100 and trypsin, the wells were then stained with Coomassie Blue to visualize the collagen cleavage. The experiments were repeated at least three times with or without *P. gingivalis* supernatant.

An inflamed cell line which cleaved all the collagen in the well (aggressive phenotype) and a healthy cell line which only cleaved the underneath collagen (non-aggressive phenotype) were selected and treated with 0.5 μg/mL of Arg-gingipain as a stimulant instead of the *P. gingivalis* supernatant. Arg-gingipain was purchased from Athens Research and Technology (Athens, GA, USA).

Statistical analyses

A chi-squared test was used to test for differences in the proportion of the tissue samples that were aggressive in the healthy and inflamed cell lines. Fisher's exact test was used to test for an association between smoking and disease status within the group of samples that cleaved aggressively, as well as between aggressive and non-aggressive phenotypes from smokers (disregarding the tissue status).

Table 1. Cell lines from healthy and diseased tissues

		Diseased	Healthy	Total
Aggressive	Number	12	5	17
	Percentage	70.58	29.41	100
Non-Aggressive	Number	9	16	25
	Percentage	38.50	62.50	100
Total		21	21	42

*There was a significant difference in the proportion of the samples that were aggressive from the healthy and diseased cell lines with a *p*-value of 0.0278.

Results

Healthy human gingival fibroblasts

The collagen in the wells was totally cleaved by five of the 21 fibroblast cell lines isolated from clinically healthy tissues in the presence of *P. gingivalis* (Table 1). These five cell lines were designated as aggressive phenotypes (Fig. 1A). Sixteen cell lines cleaved only the collagen underneath the cell colonies in the presence of *P. gingivalis* (Table 1). These 16 cell lines were designated as non-aggressive phenotypes (Fig. 1B). One of the five cell lines that cleaved all the collagen in the well was from a smoker and two of the 16 cell lines that cleaved only the collagen underneath the cell colonies were from smokers.

Inflamed human gingival fibroblasts

The collagen in the wells was totally cleaved by 12 of the 21 fibroblast cell lines isolated from the inflamed tissues in the presence of *P. gingivalis* (Table 1), which were designated as aggressive phenotypes (Fig. 2A). Nine cell lines cleaved only the collagen underneath the cell colonies in the presence of *P. gingivalis* (Table 1) and were designated as non-aggressive phenotypes (Fig. 2B). Three of the 12 cell lines that cleaved all the collagen in the wells were from smokers and none of the nine that only cleaved the collagen underneath the colonies were from smokers.

Treatment with Arg-gingipain

The collagen was totally cleaved by an aggressive phenotype cell line in the presence of Arg-gingipain, exactly as occurred in the presence of *P. gingivalis* supernatant (Fig. 3A). A non-aggressive phenotype cell line cleaved only the collagen underneath the cell colony in the presence of Arg-gingipain, exactly as occurred with the *P. gingivalis* supernatant (Fig. 3B).

Statistical analyses

There was a significant difference in the proportion of the samples that were aggressive from the healthy cell lines and the inflamed cell lines, with a *p*-value of 0.0278 (Table 1). There was no significant difference in the proportion of samples that were aggressive between smoking and diseased status, with a *p*-value of 0.999. Also, there was no significant difference in the proportion of aggressive and non-aggressive cell lines (disregarding tissue status) among smokers, with a *p*-value of 0.2017.

Discussion

In a previous study by Zhou and Windsor (11), it was demonstrated 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 lines (aggressive phenotypes). In contrast, the same treat-

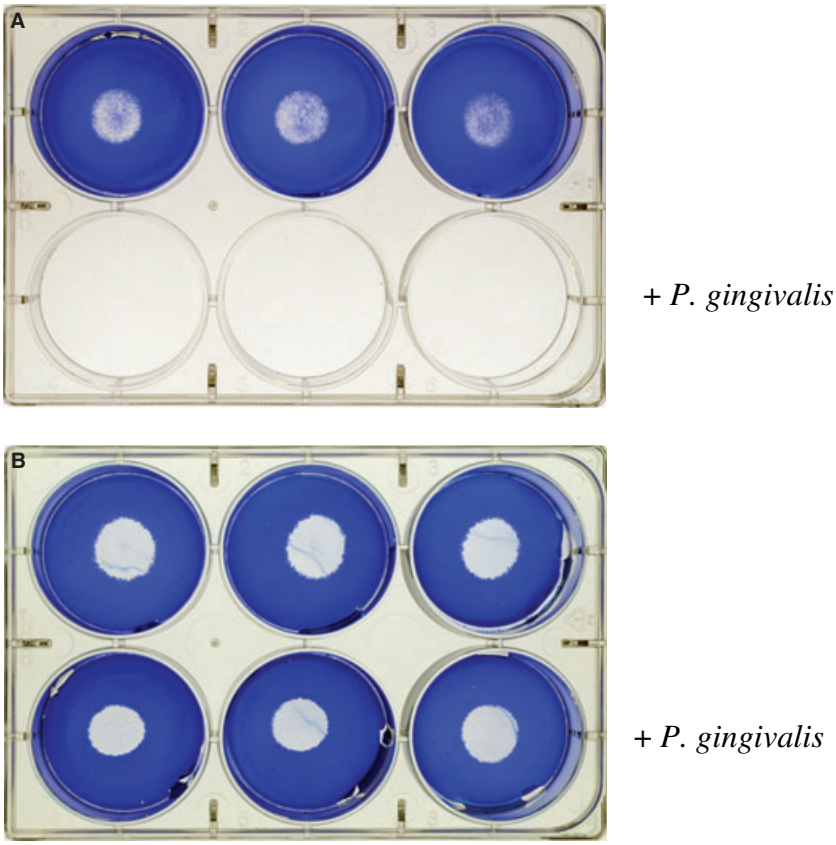
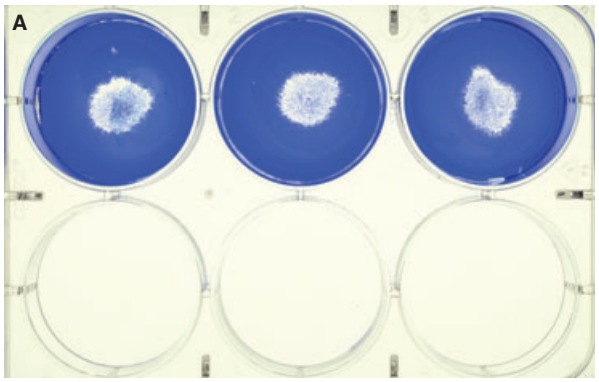


Fig. 1. Healthy human gingival fibroblasts were seeded as single colonies (50,000 cells per well) in the center of six-well plates coated with reconstituted rat-tail type I collagen. On day 6, the HGFs were removed with Triton X-100 and trypsin. The collagen cleavage was then visualized by Coomassie Blue staining. (A) Aggressive phenotype. (B) Non-aggressive phenotype.



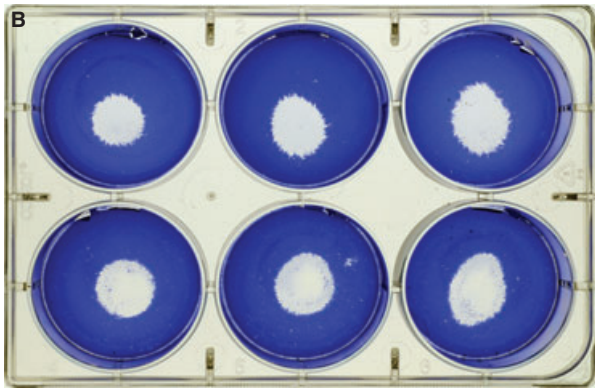


Fig. 2. Inflamed human gingival fibroblasts were seeded as single colonies (50,000 cells per well) in the center of six-well plates coated with reconstituted rat-tail type I collagen. On day 6, the human gingival fibroblasts were removed with Triton X-100 and trypsin. The collagen cleavage was then visualized by Coomassie Blue staining. (A) Aggressive phenotype. (B) Non-aggressive phenotype.

ment did not alter, or even decreased, the collagen-degrading ability of other cell lines (non-aggressive phenotype; 11). Using the same cell-mediated collagen degradation assay as Zhou and Windsor (11), the collagen-degrading ability of gingival fibroblasts isolated from inflamed and healthy tissues was compared. It was determined that there were 12 aggressive cell lines out of 21 total cell lines established from inflamed tissues and only five aggressive cell lines out of 21 total cell lines established from healthy tissues that were stimulated by the *P. gingivalis* to cleave all the collagen in the wells. *Porphyromonas gingivalis* was used as a stimulus in this study because it is one of the pathogens most often found in chronic periodontal disease sites. In addition, Zhou and Windsor (11) reported that *P. gingivalis* supernatant 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 slightly affecting the mRNA expression of multiple MMPs and TIMPs (11). These alterations could lead to the increase in ECM degradation seen in the aggressive phenotypes. Zhou and Windsor (11) found that a non-aggressive cell line had no increase in MMP activation when treated with *P. gingivalis* supernatant. Zhou and Windsor (11) concluded that the rate-limiting factor was the activation of the MMPs and not MMP expression in *P. gingivalis*-stimulated collagen degradation. Therefore, the focus of the present study was on the ability of the cell lines to respond to *P. gingivalis* stimulation.

Gingipains are cysteine proteinases that are key virulence factors produced from *P. gingivalis* (13). There are arginine (Arg)- and lysine (Lys)-specific gingipains that are cell associated and/or released. They are referred to as Arg-gingipain (Rgp) and Lys-gingipain (Kgp; 13). A number of studies (14–16) have revealed that these proteinases are closely associated with the periodontopathogenesis of *P. gingivalis*, which involves the destruction of periodontal connective tissues, disruption of host defense mechanisms, and the development and maintenance of inflammation in the periodontal pockets. Gingipains indirectly contribute to tissue damage through the activation of latent host MMPs and the inactivation of host proteinase inhibitors (17). Gingipains also enhance the expression of latent MMPs in rat mucosal epithelial cells and human fibroblasts (17). Furthermore, gingipains have been demonstrated to affect cytokine signaling networks and to modulate the production of proinflammatory mediators [interleukin (IL)-1, IL-6, IL-8, and tumor necrosis factor α], which may initiate tissue destruction and alveolar bone resorption (17).

In the present study, Arg-gingipain stimulated an inflamed aggressive cell line to cleave all the collagen in the wells, as did the *P. gingivalis* supernatant. The Arg-gingipain, like the *P. gingivalis* supernatant, did not alter the collagen cleavage mediated by a non-aggressive cell line. Therefore, this suggested that the gingipains are possibly the mediators responsible for the enhanced collagen-degrading ability of the aggressive HGF phenotypes. This is further supported by the fact that E-64 (N-[N-(L-3-transcarboxyirane-2-carbonyl)-L-Leucyl]-agmatine) inhibits the ability of *P. gingivalis* supernatant to enhance collagen degradation in an aggressive HGF cell line (18), since gingipains are cysteine proteinases that are inhibited by E-64. In addition to gingipains, which are know to be the most important virulence factors, colonization of *P. gingivalis* in the host tissue is accomplished by several other putative virulence factors (19). These include fimbriae, lipopolysaccharides, other proteases and outer membrane proteins. Abe *et al.* (20) suggested that Lys-gingipain, like Arg-gingipain, plays a crucial role as a virulence factor from *P. gingivalis* in the development of periodontal disease via the destruction of periodontal tissue components and the disruption of normal host defense mechanisms (20).

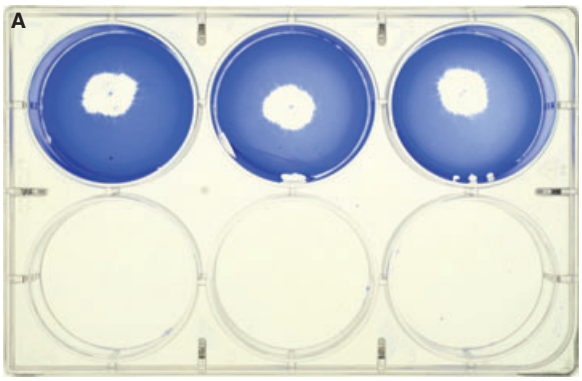


Fig. 3. Human gingival fibroblasts were seeded as single colonies (50,000 cells per well) in the center of six-well plates coated with reconstituted rat-tail type I collagen. On day 6, the human gingival fibroblasts were removed with Triton X-100 and trypsin. The collagen cleavage was then visualized by Coomassie Blue staining. (A) Aggressive phenotype. (B) Non-aggressive phenotype.

The present study included only six smokers. Three of them were in the inflamed category and three were in the healthy category. All the collagen was cleaved by all of the inflamed cell lines and one of the healthy cell lines in the presence of *P. gingivalis*. The cells from the remaining two smokers in the healthy category only cleaved the collagen underneath the cell colonies. These numbers were too small to get any statistical significance and to elucidate the possible role of smoking in this process.

A possible explanation for the differences in regard to the amount collagen cleavage in the different cell lines in the presence of *P. gingivalis* supernatant is that the cell lines might regulate the function of the mediators in the supernatant differentially. One of these mediators is most likely to be Arg-gingipain (11). Arg-gingipain is regulated by the activity of the environmental reducing–oxidative conditions. Arg-gingipain can be inactivated in an environment that contains low levels of reducing agents (11). Another possibility is there is an inhibitor of Arg-gingipain produced by the non-aggressive cell lines. However, there is no evidence to support this to date. A study by Miyachi *et al.* (21) showed that immunization with Arg-gingipain DNA vaccine is an effective method for preventing alveolar bone loss incurred by infection with *P. gingivalis* in a mouse study. Their study (21) further implicated Arg-gingipain as a major mediator in periodontal disease.

In summary, the results of this study showed that cells from diseased gingival tissues more readily cleaved collagen than cells from healthy gingival tissues and the difference was statistically significant. Also, it was demonstrated that pure Arg-gingipain gave identical results to those for *P. gingivalis* supernatant, thus demonstrating that it is one of the mediators in the *P. gingivalis* supernatant that plays a role in increasing the collagen-degrading ability of some HGFs.

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