INFECTION AND IMMUNITY, Dec. 1996, p. 4940–4945 Vol. 64, No. 12
0019-9567/96/$04.00+0

Effect of *Porphyromonas gingivalis* on Epithelial Cell MMP-9 Type IV Collagenase Production

PHILIPPE FRAVALO, CHRISTIAN MÉNARD, AND MARTINE BONNAURE-MALLET*

Equipe de Biologie Buccale EA1256, UFR Odontologie, Université de Rennes I, 35000 Rennes, France

Received 14 May 1996/Returned for modification 7 June 1996/Accepted 16 September 1996

Porphyromonas gingivalis **is reportedly capable of stimulating the expression of host cell matrix metalloproteinases (MMP), contributing to tissue destruction. However, the impact of this bacterium on specific molecules remains to be determined. In this study, we evaluate the effect of *P. gingivalis* on regulation of MMP-9 expression in human gingival epithelial cells (HGEC). Various inocula of *P. gingivalis* were added to cultures of HGEC. The effects of live bacteria, heat-killed bacteria, and outer membrane extract were analyzed. MMP-9 secretion by HGEC was evaluated by enzyme-linked immunosorbent assay. For inocula smaller than one bacterium per cell, the quantity of MMP-9 secreted by HGEC was increased in comparison to control conditions. For inocula from 2.5 to 250 bacteria per cell, an inhibition of MMP-9 secretion in a dose-response fashion was observed, with a maximum reduction (ranging from 80 to 95% in five experiments) at 50 bacteria per cell. Gelatin zymograms confirmed the decrease in MMP-9 secretion. A band of 83 kDa, corresponding to activated enzyme, was present for inocula of 0.5 to 50 bacteria. Inhibition took place without any alteration of epithelial cell viability. Heat-killed bacteria and outer membrane extract also provoked proenzyme activation but did not inhibit MMP-9 secretion. These results demonstrate a direct effect of *P. gingivalis* on HGEC, suggesting a specific action on the collagen renewal process at the interface between the epithelium and connective tissue.**

Periodontal diseases are complex, multifactorial, polymicrobial diseases characterized by the destruction of the periodontal attachment apparatus following an intense inflammatory response (reviewed in references 12, 37, 41, and 44). The components of the extracellular matrix (ECM), especially collagens, appear to be the main targets of degradation (reviewed in reference 3). Collagens normally form elements which constitute the structural basis for the cohesion and mechanical stability of the ECM. It is therefore obvious that agents that directly or indirectly modify the ECM have the potential to affect the course of periodontal disease. Many bacteria of the microbial plaque possess numerous proteinases that can potentially degrade components of the ECM of the periodontal tissues, leading to tissue destruction. However, only a few bacterial species associated with periodontal disease are considered to play a true causal role in the disease (13, 31, 41). Among these, *Porphyromonas gingivalis*, which possesses many virulence factors (9) including a trypsin-like and a collagenolytic enzyme (4, 15), and a β-*N*-acetylhexosaminidase (26), is suspected to cause direct enzymatic degradation of periodontal tissues (46). Conversely, indirect evidence indicates that metabolic degradation of the ECM is caused by a family of host-derived proteolytic enzymes known as the matrix metalloproteinases (MMP) (reviewed in reference 5). Induction or repression of expression of most MMP genes is controlled by growth factors and cytokines. A model for microbe-induced, host-mediated destruction of the tissues supporting the tooth, based on dysregulation of MMP gene expression, has been recently proposed, but many important details remain to be elucidated (3).

P. gingivalis is known to invade (reviewed in reference 11) and to persist within (27) cultured epithelial cells. Keratinocytes of human periodontal tissues, which may well be the first type of cell with which *P. gingivalis* comes into contact, produce members of the three subclasses of the MMP family. Either constitutively or after appropriate induction, keratinocytes express, in vitro, two gelatinases, MMP-2 and MMP-9, one collagenase, MMP-1, and stromelysins, MMP-3, MMP-7, MMP-10, and MMP-11 (3, 34, 35, 48), but it is not immediately apparent that these enzymes are also expressed in vivo (3). Zymography has shown that MMP-9 is the main gelatinase in oral fluids of healthy subjects and periodontitis patients, with significantly higher levels in the latter, and has therefore been implicated in tissue destruction in periodontitis (28). However, MMP-9 levels in gingival crevicular fluid are not closely associated with clinical parameters of periodontitis, including pocket depth and gingival index (43).

Since a key question concerning MMP activity in periodontal diseases is what turns it on and off, this study aimed to evaluate the direct effect of the presence of the periopathogen *P. gingivalis* on MMP-9 production by human gingival epithelial cells (HGEC). We used an in vitro culture of gingival epithelial cells (from surgical specimens) inoculated with various numbers of *P. gingivalis* cells. Further, we analyzed the effects of heat-killed bacteria and outer membrane extract by the same method. We showed that MMP-9 secretion by HGEC is inhibited by the presence of viable *P. gingivalis*. We also showed that MMP proenzyme is activated by viable bacteria and by outer membrane extract. This information suggests that *P. gingivalis* may play a specific role in the loss of connective tissue attachment in periodontal disease by local activation and down-regulation of MMP-9. A dysregulation of MMP activation and production may cause altered extracellular proteolysis and bring about a general lack of efficiency in ECM repair and reorganization.

* Corresponding author. Mailing address: Equipe de Biologie Buccale EA1256, UFR Odontologie, Université de Rennes I, 2 place Pasteur, 35000 Rennes, France. Phone: 99.63.19.55. Fax: 99 38 17 45. Electronic mail address: Martine.Bonnaure@univ-rennes1.fr.

MATERIALS AND METHODS

Primary cultures of HGEC. Primary cultures of HGEC were derived from explants of healthy human gingival tissues obtained as *res nullius* of routine surgical procedures. Surgical specimens were preserved at 4°C in a mixture

containing Dulbecco's modified Eagle medium (DMEM; BioWhittaker, Fontenay, France), 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.5), antibiotics (100 IU of penicillin G and 100 μg of streptomycin per ml), and amphotericin B (Fungizone; 5 μg/ml) until use (within 12 h). Gingival specimens were rinsed in phosphate-buffered saline (PBS) and cut into fragments of approximately 1 mm^3, visually excluding fragments appearing to be conjunctival, and grown in petri dishes coated with type I collagen from rat tail (Sigma-Aldrich Chimie, Saint Quantin Fallavier, France) in DMEM-Ham's F12 medium (1:1) supplemented with 10% heat-inactivated fetal calf serum (BioWhittaker), 2.75 μM hydrocortisone, and antibiotics (17).

Subcultures of HGEC were grown in CE medium (DMEM-Ham's F12 [3:1], 10% fetal calf serum, 1.1 μM hydrocortisone, 8.4 ng of cholera toxin per ml, 6 μg of insulin per ml, 20 ng of epidermal growth factor [Sigma-Aldrich] per ml, antibiotics) (17).

Bacterial culture. A broth culture of *P. gingivalis* W83 (obtained from Christian Mouton, Université Laval, Québec, Québec, Canada) was inoculated from an agar culture and grown anaerobically for 24 h in Todd-Hewitt broth (BBL Microsystems, AES, Combourg, France) enriched with hemin (10 μg/ml) and vitamin K (1 μg/ml). The strain was maintained on Todd-Hewitt agar enriched with 2% human laked blood. *Prevotella intermedia* and *Propionibacterium acnes* (one strain of each, of clinical origin) were grown under the same conditions. Outer membrane extracts of *P. gingivalis* were prepared by glass bead attrition in the presence of EDTA as previously described (33). Protein concentration was determined by bicinchoninic acid-copper reaction (40).

Bioassays. At the third passage, a maximal inoculum of HGEC was added to 24-well plates coated with type I collagen, grown for 24 h in CE medium, and then washed in PBS and grown in CE^- (CE^- without fetal calf serum, hydrocortisone, and antibiotics). Before experiments, the viable cells were counted after removing the medium and detaching the cells with trypsin-EDTA solution (BioWhittaker) from three wells per plate, using trypan blue exclusion on a hemacytometer. The epithelial character of the culture cell population was confirmed by morphological criteria and human cytokeratin immunohistochemistry as previously described (18). Epithelial cells grown in CE^- were used as unstimulated controls. Epithelial cells grown in CE^- containing 50 ng of 12-*O*-tetradecanoylphorbol 13-acetate (TPA; Sigma-Aldrich) per ml were used as stimulated controls (35).

The bacterial culture, diluted 1/10 in fresh Todd-Hewitt broth, was grown overnight to obtain logarithmic-phase growth. The bacteria were harvested by centrifugation at 10,000 × *g* for 5 min in a microcentrifuge, washed twice in PBS, resuspended in CE^- medium to an A_{660} corresponding to 10^8 CFU/ml, and used to inoculate HGEC cultures at a ratios of 5×10^{-3} to 5×10^2 bacteria per HGEC. In one experiment, the influence of contact time between HGEC and bacteria on MMP-9 secretion was studied; after a light agitation of the 24-well plate, supernatants were pipetted off, filtered through a 0.22-μm-pore-size filter to remove bacteria, and returned to the wells. In another experiment, HGEC were incubated for 48 h in the presence of heat-killed bacteria obtained by a 1-h incubation of a broth culture at 63°C in a water bath. Bacterial death was confirmed by culturing a sample of the broth. In the last experiment, HGEC were incubated for 48 h in culture medium containing various concentrations of an outer membrane extract of *P. gingivalis*. All experiments were performed in triplicate.

Viability of epithelial cells. The capacity for trypan blue exclusion after 48 h of mixed culture was measured by counting in a Thoma chamber after dispersion of the HGEC layer with a 0.25% trypsin–0.5 mM EDTA solution. Cell cultures were also subjected to an MTT [3-(4,5-dimethylthiazole-2-yl-2,5-diphenyl tetrazolium bromide] test (ICN Pharmaceuticals, Orsay, France) (32), where viability, characterized by the conservation of metabolic activity (mitochondrial dehydrogenase), leads to the intracellular formation of formazan crystals. This formation was monitored by inverse microscopy and quantified in 96-well microplates by optical density reading at 550 nm in a Dynatech MR5000 enzyme-linked immunosorbent assay (ELISA) reader (Dynatech, Bio-Times, Betton, France) after complete solubilization of the formazan in dimethyl sulfoxide.

Secretory activity. Changes in secretory activity were assessed by sodium dodecyl sulfate (SDS)–12% polyacrylamide electrophoresis (PAGE) as described by Laemmli (21). Prior to electrophoresis, 48-h culture supernatants were clarified by centrifugation at 10,000 × *g* for 5 min in a microcentrifuge and concentrated 10-fold in a Centricon 30 (Grace, Epernon, France). The separated proteins were stained with silver (1) and visually compared.

MMP-9 quantification by ELISA. Quantification of secreted MMP-9 was performed using a modification of a method previously described (19). The purified MMP-9, the specific antiserum, and purified antibodies against human MMP-9 were kindly provided by Lars Kjeldsen, Rigshospitalet, Copenhagen, Denmark. Briefly, Maxisorp (Nunc, Poly Labo, Strasbourg, France) microtiter plates were coated overnight at 4°C with a rabbit anti-human MMP-9 immunoglobulin G fraction diluted 1:5,000 in 50 mM carbonate buffer (pH 9.6). The plates were washed with PBS and blocked for 1 h in 8 mM phosphate buffer (pH 7.2) containing 1% bovine serum albumin (BSA), 1% Triton X-100, 3 mM KCl, and 0.5 M NaCl. The samples were added and incubated for 1 h with constant shaking. The plates were washed three times with 8 mM phosphate buffer (pH 7.2) containing 0.1% BSA, 0.1% Triton X-100, 3 mM KCl, and 0.5 M NaCl and then incubated for 1 h in biotinylated affinity-purified rabbit anti-human MMP-9 antibody diluted 1:9,000 in the same solution. The plates were washed as before, and the signal was amplified by an ABComplex system (Dako, Trappes, France) according to the manufacturer's instructions. Color was developed by a 30-min incubation in 0.1 M citric acid buffer (pH 5.0) containing 0.04% *o*-phenylenediamine and 0.03% H_2O_2 (100 μl per well), and the reaction was stopped by the addition of 100 μl of 1 M H_2SO_4. Absorbance was read at 490 nm in a Dynatech MR5000 ELISA reader. Purified MMP-9 diluted to 0.5 to 20 ng/ml was used as a standard. Culture supernatant samples were concentrated 2.5-fold. Unless otherwise mentioned, all steps were performed at room temperature.

Assays for metalloproteinase activity. Gelatin zymography (16) was used to assess metalloproteinase activity and to identify enzyme components on the basis of molecular size. Briefly, 11% polyacrylamide gels containing 0.2% gelatin, a substrate for the enzymes of interest, were prepared. Culture supernatants, clarified by centrifugation at 12,000 × *g* for 10 min and concentrated 2.5-fold were electrophoresed under denaturing but nonreducing conditions (i.e., in the presence of SDS). Samples were not boiled prior to electrophoresis. After migration at 10 mA for 3.5 h at 4°C, the gels were rinsed twice in a solution of 2.5% Triton X-100 to remove SDS. The gels were then incubated at 37°C in 50 mM Tris HCl (pH 7.5)–1 mM $CaCl_2$–0.01% NaN_3, either without protease inhibitors or with the addition of 1.25 μM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK; an inhibitor of serine proteases) and 1.0 mM 4-(2-aminoethyl)-benzenesulfonylfluoride, hydrochloride (AEBSF; an inhibitor of serine proteases), or 1.5 mM EDTA (an inhibitor of MMP). In the latter case, $CaCl_2$ was omitted from the incubation buffer. The gels were finally stained with Coomassie blue and destained in methanol-acetic acid. Lytic activity appeared as bands of negative staining.

RESULTS

***P. gingivalis* inhibits MMP-9 secretion.** The epithelial nature of the eukaryotic cell population was confirmed by the expression of cytokeratins 8, 14, 18, and 19 (data not shown). After 48 h of growth in the presence of *P. gingivalis*, secretion of MMP-9 by HGEC was evaluated by ELISA. This experiment was performed five times, on five different primary cultures of HGEC. For inocula smaller than one bacterium per cell, the quantity of MMP-9 secreted by the epithelial cells was weakly but reproducibly increased in comparison to control conditions (Fig. 1). For inocula of 2.5 to 250 bacteria per cell, we observed inhibition of MMP-9 secretion in an inverse dose-response fashion, with a maximum reduction (80 to 95% in five different experiments) at 50 bacteria per cell and a 50% inhibition at 5 bacteria per cell. When we tested the two other bacterial species (*P. intermedia* and *P. acnes*) at the maximum inoculum (500 bacteria per HGEC), no variation was observed in the quantity of MMP-9 secreted in comparison to control conditions (data not shown).

***P. gingivalis* activates MMP-9 proenzyme.** Gelatin zymography was used to assess gelatinase activity and to identify enzyme components on the basis of size. In this technique, SDS activates the proenzyme and allows visualization of the 92-kDa form of the enzyme; the active form (83 kDa) is also visualized. Zymograms confirmed the decrease in MMP-9 secretion (92-kDa gelatinase activity) and also gave qualitative information, in that a band of a size corresponding to that of the activated enzyme (83 kDa) was present for inocula of 0.5 to 50 bacteria per cell, but weakly for the latter (Fig. 1). The 83-kDa band also faded with increasing inoculum.

A 72-kDa band corresponding to gelatinase A (MMP-2) was also observed but was not quantitatively modified when the ratio increased. In addition, it was found that all gelatinase activity was completely suppressed when EDTA was included and also in the absence of $CaCl_2$ (Fig. 2). In contrast, TLCK and AEBSF, potent serine proteinase inhibitors, were without effect. These data confirm that lytic activity was associated with the MMP family.

More than 8 h of contact time between the bacteria and the cells was required to inhibit secretion (Fig. 3). However, after 30 min of contact, a modification of the 92-kDa proenzyme to the 83-kDa activated enzyme was noted on zymography (data not shown). In contrast, *P. gingivalis* gelatinolytic enzymes (such as trypsin-like enzymes) did not contribute to the lytic

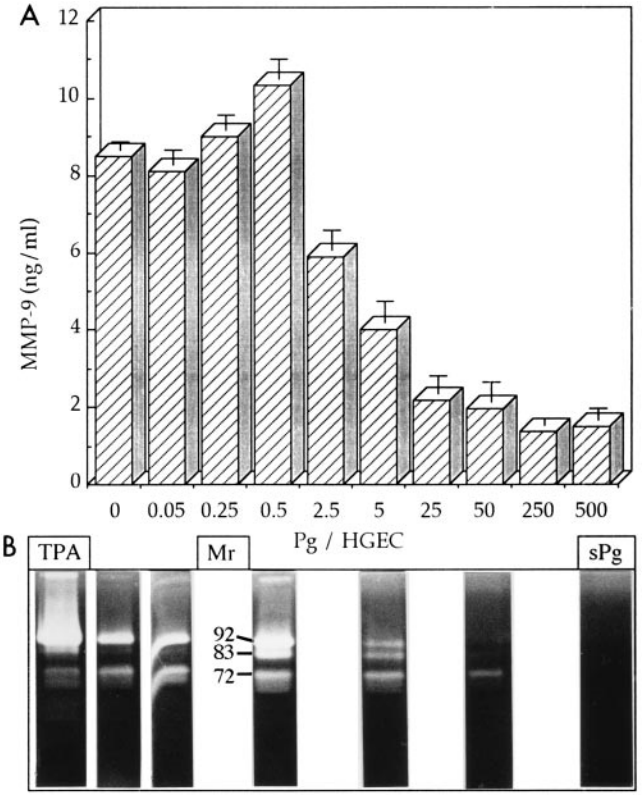

FIG. 1. Dose-response study of the effect of *P. gingivalis* (Pg) on in vitro MMP-9 secretion by HGEC. (A) Data represent mean MMP-9 secretion. Error bars represent standard errors for three wells ($n = 3$) at the same bacterial concentration as measured by ELISA. (B) Gelatin zymograms of concentrated supernatants from cultures of HGEC receiving various bacterial inocula (under the corresponding ratios in panel A), TPA (positive activated control), or the highest bacterial concentration in CE^- medium without HGEC (designated sPg). M_rs are in thousands.

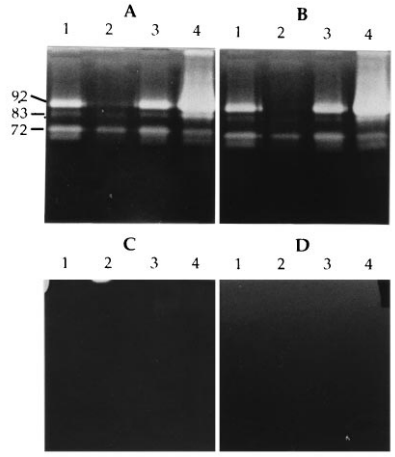

FIG. 2. Assays for metalloproteinase activity. Shown are gelatin zymograms of concentrated supernatants from HGEC cultures receiving no bacteria (lanes 1), 50 bacteria per HGEC (lanes 2), 0.05 bacteria per HGEC (lanes 3), or TPA (lanes 4). Zymograms were visualized without protease inhibitor (A), in the presence of trypsin inhibitor (TLCK) and serine proteinase inhibitor (AEBSF) (B), in the presence of metalloproteinase inhibitor (EDTA) (C), or in the absence of $CaCl_2$ (D).

banding pattern on zymography, since no lytic band could be observed from the 48-h supernatant of *P. gingivalis* (equivalent to 50 bacteria per cell) grown alone in the CE^- medium (Fig. 1).

Is *P. gingivalis* viability essential to inhibit MMP-9 secretion? Preliminary experiments on the viability of bacteria grown alone in CE^- medium showed 95% of the initial CFU after 8 h of culture, 80% after 25 h, and 62% after 49 h. These results demonstrated that *P. gingivalis* tolerates oxygen when grown in liquid medium. When HGEC were cultured for 48 h with heat-killed *P. gingivalis*, no significant difference was observed in MMP-9 secretion. However, heat-killed bacteria provoked proenzyme activation in the same ratio range as live bacteria (data not shown). A 1-h incubation of bacteria at 63°C is sufficient to kill the bacteria, but most proteins (including enzymes) are only reversibly denatured.

In an additional set of experiments, HGEC were grown with various amounts of outer membrane extract from *P. gingivalis* to determine the role of this bacterial cell fraction in inhibition and activation of MMP-9. After 48 h of culture, activation of the 92-kDa enzyme to the 83-kDa form was observed in zymography for concentrations of greater than 1 μg/ml (Fig. 4), but no significant decrease in MMP-9 secretion was noted by ELISA (data not shown).

HGEC viability. After 48 h of growth, viability of HGEC was over 95%, as judged both by trypan blue exclusion and by intracellular formation of formazan crystals (MTT test), regardless of stimulation by TPA or incubation in the presence of bacteria (Table 1). However, after 48 h, the metabolic activity of experimental cells did not exceed 75% of that of control cells taken as a reference (100%).

To determine whether the general metabolic activity was altered, a preliminary analysis of general secretory activity was done by SDS-PAGE. Silver staining of culture supernatant revealed very little qualitative change in the profile of secretory products, suggesting the absence of any degradation of these products even at 50 bacteria per HGEC. An increase and a decrease in total secretory products were observed in the lanes representing TPA and 50 bacteria per HGEC, respectively (Fig. 5), which both retained 75% metabolic activity (Table 1).

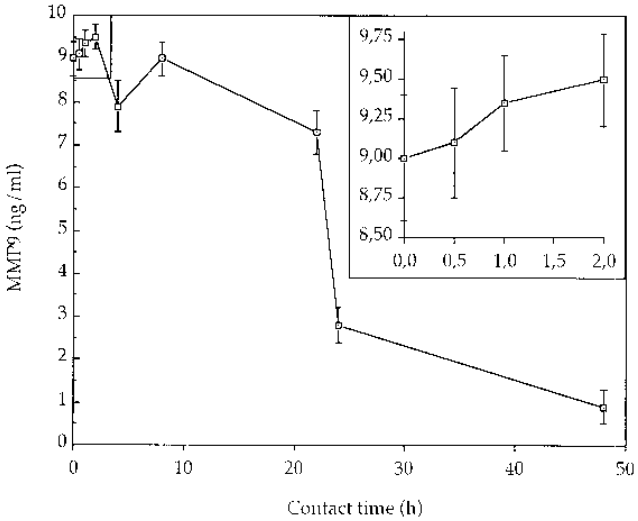

FIG. 3. Kinetics of MMP-9 production for HGEC grown with *P. gingivalis* during different contact times. MMP-9 was measured in culture supernatants recovered up to 48 h after different contact times with 50 bacteria per HGEC. Error bars represent standard deviations ($n = 3$). The inset shows kinetics for the first 2 h in detail.

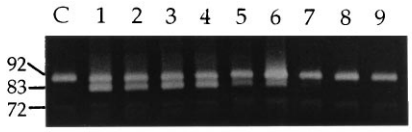

FIG. 4. Zymograms of 10-times-concentrated supernatants from HGEC incubated with various concentrations of outer membrane extract instead of bacteria. Lanes: C, control conditions without extract; 1 to 9, outer membrane extract at 12.5, 10, 8, 6, 5, 2.5, 1, 0.1, and 0.01 μg/ml. Sizes are indicated in kilodaltons.

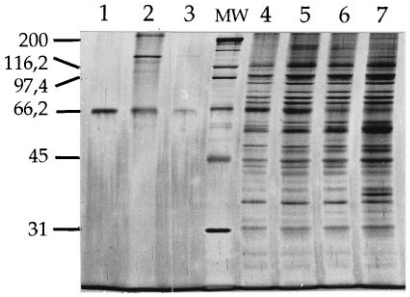

FIG. 5. Silver-stained SDS-polyacrylamide gel of concentrated supernatant from culture medium alone (lane 1), the highest bacterial concentration in medium without HGEC (lane 2), TPA in medium without HGEC (lane 3), 50 bacteria per HGEC in medium (lane 4); 0.5 bacterium per HGEC in medium (lane 5), medium with HGEC (lane 6), and TPA in medium with HGEC (lane 7). Molecular weights (MW) are indicated in thousands.

DISCUSSION

Evidence has accumulated that *P. gingivalis* is a member of the commensal flora of the oral cavity (16, 25, 29, 30, 39), suggesting that its role in periodontitis is largely opportunistic (25, 30). These findings do not necessarily weaken the evidence for a decisive role for *P. gingivalis* in periodontal disorders. However, to play a role in a multifactorial, polymicrobial infection, *P. gingivalis* should demonstrate specific properties allowing it to shift from commensal to pathogenic action. Such properties may imply molecular interactions between *P. gingivalis* and a key target of the periodontium, the gingival epithelial cell.

It is difficult to design a biologically relevant in vitro study of a pathological process. The model that we used in this study aimed to answer the very simple question of whether *P. gingivalis* modifies the production of MMP-9 by HGEC. As stated by Lamont et al. (23), HGEC are the progenitor cells of those found on the surface of the gingiva and constitute a more relevant model for an investigation of the interactions between bacteria and the gingival crevice than do buccal or other epithelial cells. Using this model, we observed, in preliminary unpublished work, a maximal internalization of *P. gingivalis* at an inoculum level of 50 bacteria per HGEC, which is consistent with previous findings by Lamont et al. (22). Results from the present study confirm the lack of toxicity of *P. gingivalis* for defined conditions of inoculation of a 48-h culture of HGEC, as demonstrated by Shah et al. (38) for human epithelial cell lines at a contact time of less than 24 h.

ELISA using polyclonal antibodies against MMP-9 permitted us to quantify levels of the zymogen form (92 kDa) as well as these of the activated form (83 kDa), and possibly of partially degraded forms of MMP-9. However the measurement of the 83-kDa form in comparison to the 92-kDa form should be weaker by 10 to 15% because of the lower number of epitopes recognized by polyclonal antibodies. The secretion of MMP-9 under control conditions was comparable to secretion stimulated by the addition of epidermal growth factor. However, in the mixed culture experiment, the direct interaction between *P. gingivalis* and the gingival epithelial cells was not neutral. The metabolism of the epithelial cells was reduced by 25%, and the profile of secretory products changed. More specifically, this study shows that the presence of live *P. gingivalis* at a concentration of 25 bacteria per HGEC almost completely inhibited the secretion of MMP-9. *P. gingivalis* possesses a plethora of proteases that can be secreted as free molecules or in vesicles or retained in the outer membrane (14); several of these proteases can provoke a limited cleavage and activation of MMP-1, -8, and -9 (10, 42). However, three observations argue against a possible degradative action on MMP-9 by these enzymes at the bacterium/HGEC ratio that we used in our experiments. First, 10-fold-concentrated supernatant of *P. gingivalis* culture, when loaded on a zymogram, failed to demonstrate any gelatinase activity (Fig. 1, lane sPg), despite the presence of high-molecular-weight products in silver staining of the same supernatant (Fig. 5, lane 2). Second, silver staining of HGEC secretory products showed no qualitative alteration in profiles (Fig. 5). Third, the best proof is in Fig. 1A, which showed the presence of MMP-2 at 72 kDa. There is no reason why this molecule would be protected against a potential proteolytic action from *P. gingivalis*. Thus, the presence of MMP-2 decreased only slightly from a ratio of 0.5 bacterium per HGEC to 5 bacteria per HGEC, whereas the two forms of MMP-9 were highly reduced. These results seem to contradict those by Birkedal-Hansen et al. (6), Ding et al. (10), and Uitto et al. (45), in which indirect evidence suggested that *P. gingivalis* up-regulates MMP expression.

TABLE 1. Viability of HGEC in different culture conditions, evaluated by different methods

48-h culture conditions	Mean % ± SD (n = 3)		
	Viability by trypan blue	HGEC with formazan crystals	MTT test
CE^- medium	97 ± 1	98 ± 1	100 ± 5
CE^- + TPA	95 ± 2	98 ± 1.5	76 ± 4
CE^- + 0.5 bacteria/HGEC	96 ± 1	96 ± 2	75 ± 5
CE^- + 50 bacteria/HGEC	95 ± 5	97 ± 1	78 ± 6

At the simulated infective dose of 25 bacteria per HGEC, the inhibition of secretion of this metalloproteinase required a contact time of more than 8 h. This leaves one to believe that there may be a different independent phenomenon of internalization that is optimal at 90 to 120 min and that other mechanisms come into play. However, a possible connection between the disappearance of MMP-9 production and the persistence and multiplication of the bacteria within HGEC cannot be ruled out. In fact, a decrease in the number of intracellular bacteria was reported during the first 24 h postinternalization (27) followed by a sharp increase during the next day. In five experiments, we observed only a partial inhibition (10 to 20% reduction) of MMP-9 production during the first 20 h.

Zymography adds an element to the quantification by ELISA of secretory MMP-9. The presence of the bacteria, live or dead, for a short contact time (0.5 h) led to a modification in the MMP-9 present in the supernatant of the epithelial cells. Activation was also caused by addition of *P. gingivalis* outer membranes to HGEC culture and appeared to be linked to interactions between HGEC and molecules of the outer mem-

brane. There is a difference in the rates at which the two enzyme forms will degrade gelatin, the rate for the 83-kDa form being higher than that of the 92-kDa form. Thus, since the amount of active gelatinase relative to latent gelatinase may be very much higher than that measured by the zymography, only a small part of the proenzyme was converted, suggesting that the activating bacterial molecule (presumably an enzyme) is not really effective or dedicated to this function.

MMPs are essential actors in normal tissue remodeling. Due to their potential hazardous effects, the activity of MMPs is highly regulated at four gates (5): (i) by transcriptional regulation of MMP genes, (ii) by precursor activation, (iii) by differences in substrate specificity, and (iv) by MMP inhibitors. We believe that *P. gingivalis* dysregulates the activity of MMP-9 by short-circuiting two gates of regulation. First, *P. gingivalis* may activate MMP-9 proenzyme and thus short-circuit the inhibition of this collagenase by α2-macroglobulin and tissue inhibitor of metalloproteinase, inhibitors that are both present in high concentrations in crevicular fluid (8, 20, 24, 36). Second, *P. gingivalis* short-circuits the transcriptional regulation. We hypothesized that a metabolite of the bacteria acts on HGEC in a manner similar to retinoids, which are known to downregulate MMP expression in stromal cells (7) and in epidermal keratinocytes (2), with an ambiguous effect on tissue destruction, as they stimulate rather than suppress keratinocyte-mediated collagen degradation (47). The proximity of viable microbial plaque creates an unequaled opportunity for such direct transcriptional effects.

The result is paradoxical; a dysregulation of MMP production and activation may cause altered extracellular proteolysis and bring about a widespread lack of efficiency in ECM repair and reorganization. The present results suggest that *P. gingivalis* may play a specific role in the loss of connective tissue attachment in the periodontal disease by local activation and down-regulation of MMP-9. A molecular study of differential gene expression will allow the specificity of such a response to be established and help to explain the mechanisms which give rise to it.

ACKNOWLEDGMENTS

We thank D. Ní Eidhin for editorial assistance. We especially thank Lars Kjeldsen (Rigshospitalet, Copenhagen, Denmark) for providing purified MMP-9 and anti-MMP-9 antibodies and G. Bader for providing surgical specimens used in this study.

This work was supported by CRB grant and a scholarship from MESR to P.F.

REFERENCES

1. **Ansorge, W.** 1985. Fast and sensitive detection of protein and DNA bands by treatment with potassium permanganate. J. Biochem. Biophys. Methods **11:** 13–20.
2. **Bailly, C., S. Drèze, D. Asselineau, B. Nusgens, C. M. Lapière, and M. Darmon.** 1990. Retinoic acid inhibits the production of collagenase by human epidermal keratinocytes. J. Invest. Dermatol. **94:**47–51.
3. **Birkedal-Hansen, H.** 1994. Host-mediated extracellular matrix destruction by metalloproteinases, p. 191–202. *In* R. Genco, S. Hamada, T. Lehner, J. McGhee, and S. Mergenhagen (ed.), Molecular pathogenesis of periodontal disease. American Society for Microbiology, Washington, D.C.
4. **Birkedal-Hansen, H., R. E. Taylor, J. J. Zambon, P. K. Barwa, and M. E. Neiders.** 1988. Characterization of collagenolytic activity from strains of *Bacteroides gingivalis*. J. Periodont. Res. **23:**258–264.
5. **Birkedal-Hansen, H., R. E. Taylor, J. J. Zambon, P. K. Barwa, and M. E. Neiders.** 1993. Role of matrix metalloproteinases in human periodontal diseases. J. Periodont. Res. **64:**474–483.
6. **Birkedal-Hansen, H., B. R. Wells, H. Y. Lin, P. W. Caufield, and R. E. Taylor.** 1984. Activation of keratinocytes-mediated collagen (type I) breakdown by suspected periopathogens: evidence of a novel mechanism of connective tissue breakdown. J. Periodont. Res. **19:**645–650.
7. **Brinckerhoff, C. E., J. W. Coffey, and A. C. Sullivan.** 1983. Inflammation and collagenase production in rats with adjuvant arthritis reduced with 13-cis-retinoic acid. Science **221:**756–758.
8. **Condacci, I., G. Cimasoni, and C. Ahmad-Zadeh.** 1982. α2-Macroglobulin in sulci from healthy and inflamed human gingivae. Infect. Immun. **36:**66–71.
9. **Cutler, C. W., J. R. Kalmar, and C. A. Genco.** 1995. Pathogenic strategies of the oral anaerobe, *Porphyromonas gingivalis*. Trends Microbiol. **3:**45–51.
10. **Ding, Y., V.-J. Uitto, J. Firth, T. Salo, M. Haapasalo, Y. T. Konttinen, and T. Sorsa.** 1995. Modulation of host matrix metalloproteinases by bacterial virulence factors relevant in human periodontal diseases. Oral Dis. **1:**279–286.
11. **Fives-Taylor, P. M., D. H. Meyer, P. K. Sreenivasan, and K. P. Mintz.** 1994. Invasion of cultured epithelial cells by periodontopathogens, p. 57–68. *In* R. Genco, S. Hamada, T. Lehner, J. McGhee, and S. Mergenhagen (ed.), Molecular pathogenesis of periodontal disease. American Society for Microbiology, Washington, D.C.
12. **Genco, R. J.** 1992. Host responses in periodontal diseases: current concepts. J. Periodontol. **63:**338–355.
13. **Genco, R. J., J. J. Zambon, and L. A. Christersson.** 1988. The origin of periodontal infections. Adv. Dent. Res. **2:**245–259.
14. **Grenier, D., G. Chao, and B. C. McBride.** 1989. Characterization of sodium dodecyl sulfate-stable *Bacteroides gingivalis* proteases by polyacrylamide gel electrophoresis. Infect. Immun. **57:**95–99.
15. **Grenier, D., and D. Mayrand.** 1993. Proteinases of *Porphyromonas gingivalis*, p. 227–243. *In* H. Shah, D. Mayrand, and R. J. Genco (ed.), Biology of the species *Porphyromonas gingivalis*. CRC Press, Boca Raton, Fla.
16. **Heussen, C., and E. B. Dowdle.** 1980. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. Anal. Biochem. **102:**196–202.
17. **Hibbs, M. S., K. A. Hasty, J. M. Seyer, A. H. Kang, and C. L. Mainardi.** 1985. Purification and characterization of the secreted forms of human neutrophil gelatinase. J. Biol. Chem. **260:**2493–2500.
18. **Huard, A., A. Corlu, A. Joffre, H. Magloire, and M. Bonnaure-Mallet.** Reconstituted human gingival epithelium: non-submerged *in vitro* model. In Vitro Cell. Dev. Biol., in press.
19. **Kjeldsen, L., O. W. Bjerrum, D. Hovgaard, A. H. Johnsen, M. Sehested, and N. Borregaard.** 1992. Human neutrophil gelatinase: a marker for circulating blood neutrophils. Purification and quantitation by enzyme linked immunosorbent assay. Eur. J. Haematol. **49:**180–191.
20. **Kryshtalskyj, E., and J. Sodek.** 1987. Nature of collagenolytic enzyme and inhibitor activities in crevicular fluid from healthy and inflamed periodontal tissues of beagle dogs. J. Periodont. Res. **22:**264–269.
21. **Laemmli, U. K.** 1970. Cleavage of natural proteins during the assembly of bacteriophage T4. Nature (London) **227:**680–685.
22. **Lamont, R. J., A. Chan, C. M. Belton, K. T. Izutsu, D. Vasel, and A. Weinberg.** 1995. *Porphyromonas gingivalis* invasion of gingival epithelial cell. Infect. Immun. **63:**3878–3885.
23. **Lamont, R. J., D. Oda, R. E. Persson, and G. R. Persson.** 1992. Interaction of *Porphyromonas gingivalis* with epithelial cells maintained in culture. Oral Microbiol. Immunol. **7:**364–367.
24. **Larivée, J., J. Dodek, and J. M. Ferrier.** 1986. Collagenase and collagenase inhibitor activities in crevicular fluid of patients receiving treatment for localized juvenile periodontitis. J. Periodont. Res. **21:**702–715.
25. **Loos, B. G., D. W. Dyer, T. S. Whittam, and R. K. Selander.** 1993. Genetic structure of populations of *Porphyromonas gingivalis* associated with periodontitis and other oral infections. Infect. Immun. **61:**204–212.
26. **Lovatt, A., and I. S. Roberts.** 1995. Cloning and expression in *Escherichia coli* of the *nahA* gene from *Porphyromonas gingivalis* indicates that β-*N*-acetylhexosaminidase is an outer membrane-associated lipoprotein. Microbiology **140:**3399–3406.
27. **Madianos, P. N., P. N. Papapanou, U. Nannmark, G. Dahlen, and J. Sandros.** 1996. *Porphyromonas gingivalis* FDC381 multiplies and persists within human oral epithelial cells in vitro. Infect. Immun. **64:**660–664.
28. **Mäkelä, M., T. Salo, V. J. Uitto, and H. Larjava.** 1994. Matrix metalloproteinases (MMP-2 and MMP-9) of the oral cavity: cellular origin and relationship to periodontal status. J. Dent. Res. **73:**1397–1406.
29. **McClellan, D. L., A. L. Griffen, and E. J. Leys.** 1995. Age and prevalence of *Porphyromonas gingivalis* in children. J. Dent. Res. **74:**252.
30. **Ménard, C., and C. Mouton.** 1995. Clonal diversity of the taxon *Porphyromonas gingivalis* assessed by random amplified polymorphic DNA fingerprinting. Infect. Immun. **63:**2522–2531.
31. **Moore, W. E. C.** 1987. Microbiology of periodontal disease. J. Periodont. Res. **22:**335–341.
32. **Mosmann, T.** 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods **65:**55–63.
33. **Parent, R., C. Mouton, L. Lamonde, and D. Bouchard.** 1986. Human and animal serotypes of *Bacteroides gingivalis* defined by crossed immunoelectrophoresis. Infect. Immun. **51:**909–918.
34. **Petersen, M. J., D. T. Woodley, G. P. Stricklin, and E. J. O'Keefe.** 1987. Production of procollagenase by culture human keratinocytes. J. Biol. Chem. **262:**835–840.
35. **Petersen, M. J., D. T. Woodley, G. P. Stricklin, and E. J. O'Keefe.** 1989. Constitutive production of procollagenase and tissue inhibitor of metallo-

proteinases by human keratinocytes in culture. J. Invest. Dermatol. **92:**156–159.
36. **Schenkein, H. A., and R. J. Genco.** 1977. Gingival fluid and serum in periodontal diseases. I. Quantitative study of immunoglobulins, complement components, and other plasma proteins. J. Periodontol. **48:**772–777.
37. **Seymour, G. J., E. Gemmell, R. A. Reinhardt, J. Eastcott, and M. A. Taubman.** 1993. Immunopathogenesis of chronic inflammatory periodontal disease: cellular and molecular mechanisms. J. Periodont. Res. **28:**478–486.
38. **Shah, H. N., S. E. Gharbia, and C. M. O'Toole.** 1992. Assessment of the relative cytotoxicity of *Porphyromonas gingivalis* cells, products, and components on human epithelial cell lines. J. Periodontol. **63:**44–51.
39. **Sixou, J.-L., M. Bonnaure-Mallet, and C. Mouton.** 1995. Serum antibodies to *Porphyromonas gingivalis* in children. J. Periodontol. **66:**57–64.
40. **Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk.** 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. **150:**76–85.
41. **Socransky, S. S., and A. D. Haffajee.** 1992. The bacterial etiology of destructive periodontal disease: current concepts. J. Periodontol. **63:**322–331.
42. **Sorsa, T., T. Ingman, K. Suomalainen, M. Haapasalo, Y. T. Konttinen, O. Lindy, H. Saari, and V.-J. Uitto.** 1992. Identification of proteases from periodonthogenic bacteria as activators of latent human neutrophil and fibroblast-type interstitial collagenases. Infect. Immun. **60:**4491–4495.
43. **Teng, Y. T., J. Dodek, and C. A. G. McCulloch.** 1992. Gingival crevicular fluid gelatinase and its relationship to periodontal disease in human subjects. J. Periodont. Res. **27:**544–552.
44. **Tonetti, M. S.** 1993. Etiology and pathogenesis, p. 54–89. *In* N. P. Lang and T. Karring (ed.), Proceedings of the 1st European Workshop on Periodontology. Quintessence Publishing Co., New York.
45. **Uitto, V.-J., H. Larjava, J. Heino, and T. Sorsa.** 1989. A protease of *Bacteroides gingivalis* degrades cell surface and matrix glycoproteins of cultured gingival fibroblasts and induces secretion of collagenase and plasminogen activator. Infect. Immun. **57:**213–218.
46. **Uitto, V. J., K. Suomolainen, and T. Sorsa.** 1990. Salivary collagenase. Origin, characteristics and relationship to periodontal health. J. Periodont. Res. **25:**135–142.
47. **Wells, B. R., and H. Birkedal-Hansen.** 1985. Retinoic acid stimulates degradation of interstitial collagen fibrils by rat mucosal keratinocytes *in vitro*. J. Dent. Res. **64:**1186–1190.
48. **Woodley, D. T., T. Kalebic, R. J. Banes, W. Link, M. Pruniers, and L. Liotta.** 1987. Adult human keratinocytes migrating over nonviable dermal collagen produce collagenolytic enzymes that degrade type I and type IV collagen. J. Invest. Dermatol. **86:**418–423.

Editor: J. R. McGhee