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Neutrophils alter epithelial response to *Porphyromonas gingivalis* in a gingival crevice model

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Summary

A gingival crevice model (epithelial cell- *Porphyromonas gingivalis* – neutrophil) was established and used to profile gingipain, matrix metalloproteinase, MMP mediators (NGAL and TIMP-1) and cytokine networks. Smoking is the primary environmental risk factor for periodontitis. Therefore, the influence of cigarette smoke extract (CSE) was also monitored in the same model. *P. gingivalis* alone induced low levels of IL-1 β and IL-8 from epithelial cells, but high levels of both cytokines were produced on the addition of neutrophils. CSE-exposure (100 and 1000 ng/ml nicotine equivalency) significantly compromised *P. gingivalis*-induced cytokine secretion (both $p < 0.05$). *P. gingivalis* induced impressive secretion of NGAL ($p < 0.05$) which was not influenced by CSE. The influence of CSE on gingipains production was strain-specific. Purified gingipains effectively and rapidly degraded both TIMP-1 and MMP-9. Induction of large amounts of NGAL, degradation of TIMP-1, and increased gingipain activity would each be expected to prolong collagen degradation and promote disease progression. However, gingipains also degrade MMP-9. Thus, *P. gingivalis* exerts a complex influence on the proteolytic balance of a gingival crevice model. CSE-exposure reduces the pro-inflammatory cytokine burden, which may be expected to promote *P. gingivalis* survival. In addition to novel findings that provide mechanistic insight into periodontal disease progression, these results are in keeping with the recognized clinical dogma of decreased inflammation / increased disease in smokers. Thus, this straightforward gingival crevice model is established as a suitable vehicle for the elucidation of mechanisms that contribute to susceptibility to periodontitis.

Keywords

cytokines; epithelial cells; neutrophils; matrix metalloproteinases; periodontitis; *P. gingivalis*; tobacco smoke

Introduction

Periodontitis is a common infectious, chronic inflammatory disease of the supportive structures of the teeth (Detert *et al.*, 2010, Tonetti, 2009). Furthermore, multiple serious

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systemic sequelae are associated with the establishment of destructive periodontal mucosal lesions, including vascular and pulmonary diseases, pre-term birth, rheumatoid arthritis and diabetes complications (Scannapieco *et al.*, 2010). Histopathological studies and clinical assays have established that, while some periodontal pathogens can invade epithelial cells or cause low level bacteremia (Saito *et al.*, 2009, Castillo *et al.*, 2011), plaque bacteria are, essentially, confined within the gingival crevice where a high density of functional neutrophils are recruited and form a wall between the dental biofilm and the junctional epithelium (Galicía *et al.*, 2009, Scott and Krauss, 2011). Despite this, most prior studies of *P. gingivalis*-host interactions have included epithelial cells or leukocytes, but not both, our own work included (Sandros *et al.*, 2000, Bagaitkar *et al.*, 2009, Rehani *et al.*, 2008).

In order to increase relevance, a multilayer epithelial cell model has been established with which to observe *P. gingivalis* – host interactions (Dickinson *et al.*, 2011). We have recently described a gingival crevice model in which primary human neutrophils can protect human gingival epithelial cells (HGECS) from *P. gingivalis*-induced apoptosis with the neutrophils themselves exhibiting prolonged longevity (Galicía *et al.*, 2009). The protection afforded to epithelial cells was hypothesized to be partly due to efficient phagocytosis and bacterial killing by neutrophils. Furthermore, bacteria-induced cytokine production, particularly IL1 β and IL-8, was increased in the tripartite model relative to the combined cytokine output of epithelial cells and neutrophils stimulated with *P. gingivalis* separately, suggesting a synergistic innate response (Galicía *et al.*, 2009).

Tobacco use may be responsible for the majority of cases of adult periodontitis in the United States (Tomar and Asma, 2000). Smoking leads to a suppression of key inflammatory cytokines in gingival tissues and fluids (Palmer *et al.*, 2005) and the neutrophilic oxidative burst (Palmer *et al.*, 2005, Xu *et al.*, 2008), which are thought to contribute to the established phenomena of reduced overt inflammation and increased susceptibility to infection with *P. gingivalis* and other periodontal pathogens in smokers relative to non-smokers (Palmer *et al.*, 2005, Rehani *et al.*, 2008). Concomitantly, a tobacco-induced endogenous protease-antiprotease imbalance (Palmer *et al.*, 2005, Gursoy *et al.*, 2010) aligned with virulence mechanisms of established plaque bacteria, such as the gingipains of *P. gingivalis* (Guo *et al.*, 2010), are likely to contribute to the progression of periodontitis in smokers. Of the endogenous proteases, MMPs – the therapeutic target of sub-antimicrobial doses of tetracyclines - are considered of prime importance (Gu *et al.*, 2011).

Therefore, we assessed, in a tripartite gingival crevice model, (a) the production of representative pro-inflammatory cytokines (IL-1 β and IL-8); MMPs (MMP-8 and -9); and MMP-modulators (TIMP-1 and NGAL), (b) the effect of cigarette smoke extract (CSE) on these key mediators of inflammation and thus, (c) set out to establish the relevance of a crevicular model with respect to established *in vivo* phenomena, in order to permit future mechanistic studies. The influence of CSE on *P. gingivalis*-derived gingipains was also assessed.

Materials and Methods

Materials

P. gingivalis strains W83, HG66 and ATCC 33277 were purchased from the American Type Culture Collection (Manassas, VA). Unless otherwise stated, ATCC 33277 was used in all experiments. Gifu Anaerobe Medium [GAM] came from Nissui Pharmaceutical (Tokyo). 2-aminoethanol, amphotericin B (fungizone), insulin, 2-mercaptoethanol, penicillin/streptomycin, Sephadex G-150, sodium selenite, transferrin and Na-p-tosyl-L-lysine chloromethyl ketone (TLCK) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Collagen-coated plates were purchased from BD Biosciences (San Jose, CA). Keratinocyte

serum-free medium (KSFM), supplements including bovine pituitary extract, 4-12% NuPage Novex Bis-Tris minigels and Sypro® ruby protein stain came from Invitrogen (Carlsbad, CA). Precision Plus prestained protein standards were bought from Bio-Rad Life Science (Hercules, CA). Acetone, culture plates, dextran and lymphocyte separating medium came from Fisher Scientific (Suwanee, GA). Standard 3R4F reference cigarettes were obtained from Kentucky Tobacco Research and Development Center (Lexington, KY). IL-1 β and IL-8 ELISA kits were purchased from eBioscience (San Diego, CA) and Cell Sciences (Canton, MA), respectively, while MMP-8, MMP-9, NGAL and TIMP-1 ELISAs, as well as rMMP-9 and rTIMP-1, came from R&D Systems (Minneapolis, MN). Gingipain substrates (Rgp: N- α -benzoyl-DL-arginine-p-nitroanilide [BAPNA]; Kgp: acetyl-lysine-p-nitroanilide [ALNA]) were purchased from Sigma and Bachem America, Inc. (Torrance, CA), respectively.

Bacterial culture

P. gingivalis was grown in Gifu Anaerobic Medium (GAM) anaerobically (80% N₂, 10% H₂, 10% CO₂) at 37°C in a Coy Laboratories anaerobic chamber (Grass Lake, MI), harvested at mid-log phase by centrifugation, washed three times in PBS (pH 7.4), and used immediately.

Gingival epithelial cell isolation and culture

Primary human gingival epithelial cells (HGECS) were isolated from trypsinized, periodontally healthy gingival tissue biopsies obtained from patients undergoing crown-lengthening procedures, as previously described (Guggenheim *et al.*, 2009). Informed and written consent of tissue donors, as approved by the University of Louisville, Institutional Review Board, Human Subjects Protection Program (Study # 619-03) was obtained. HGECS were seeded in 60-mm plastic tissue culture plates coated with type-I collagen and incubated in 5% CO₂ at 37°C using KSFM medium containing 10 μ g ml⁻¹ of insulin, 5 μ g ml⁻¹ of transferrin, 10 μ M of 2-mercaptoethanol, 10 μ M of 2-aminoethanol, 10 mM of sodium selenite, 50 μ g ml⁻¹ of bovine pituitary extract, 100 units ml⁻¹ of penicillin/streptomycin and 50 ng ml⁻¹ of fungizone (complete medium). Antibiotic-free medium was used in all experiments involving the use of bacteria.

Conditioning of growth media

CSE-conditioned growth media (GAM-CSE for prokaryotes and KSFM-CSE for eukaryotes) were prepared by pulling cigarette smoke from standard 3R4F reference cigarettes through medium in 35 ml “drags” every 20 seconds, as previously described (Bagaitkar *et al.*, 2009). Media was sterile filtered (0.22 μ m); diluted to the required concentration in nicotine equivalents (0, 100 and 1000 ng/ml), determined by gas-liquid chromatography, as previously described (Fraser *et al.*, 2001); and pH adjusted to pH 7.2. Nicotine equivalency at physiological concentrations (Fraser *et al.*, 2001) is assumed to similarly dilute the multiple other soluble components of cigarette smoke to relevant levels.

Isolation of primary human neutrophils

Blood was collected from healthy donors by venipuncture, as approved by the University of Louisville, Institutional Review Board, Human Subjects Protection Program (Study # 619-03 and #503.05), and anti-coagulated with acid citrate dextrose. Neutrophils were isolated using lymphocyte separating medium and dextran sedimentation. Neutrophil preparations were routinely >98% pure and >95% viable.

Epithelial - neutrophil - *P. gingivalis* interactions

HGEC - neutrophil - *P. gingivalis* interactions were progressed as previously described (Galicía et al., 2009). HGEC monolayers (1×10^6 cells; fourth passage) were overlaid with viable neutrophils (1×10^6 cells) and placed back in the incubator for 30 mins. Live *P. gingivalis* (1×10^8 cells) in KSFM or KSFM supplemented with CSE (100 and 1000 ng/ml nicotine equivalents) were added to the HGEC-neutrophil combination for 24 hours.

Cytokine profiling

Cytokine (IL-1 β and IL-8) concentrations in 24 hr cell-free supernatants were measured by ELISA, according to the manufacturer's instructions. Plates were read at the appropriate absorbance using a Victor³ 1420 microplate reader (PerkinElmer, Waltham, MA).

Profiling of matrix metalloproteinases and regulators

MMP-8, -9, NGAL and TIMP-1 concentrations in 24 hr cell-free supernatants were measured by ELISA, according to the manufacturer's instructions. Plates were read at the appropriate absorbance using a Victor³ 1420 microplate reader.

Gingipain profiling

P. gingivalis cultures grown in either GAM or GAM-CSE were normalized for cell number (10^9 cells/ml) using sterile media. Cell-free supernatants were removed and corresponding cell pellets re-suspended in buffer supporting gingipain activity (0.2M Tris-HCl, 0.1M NaCl, 5mM CaCl₂, 10 mM L-cysteine, pH 7.6). Rgp and Kgp activities in cell-free supernatant or associated with whole bacterial cells were determined by enzymatic hydrolysis of N- α -benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) or acetyl-lysine-*p*-nitroanilide (ALPNA) at 1mM final concentration with absorbance measured at 405 nm after 3 hr, as we have previously described (Potempa and Nguyen, 2007), using a Victor³ 1420 microplate reader.

Purification of Rgp and Kgp gingipains

High molecular mass gingipain R (HRgpA) and gingipain K (Kgp) were purified from cell-free medium of *P. gingivalis* HG66, and RgpB from *P. gingivalis* W83, by acetone precipitation, size-exclusion chromatography using Sephadex G-150, and affinity chromatography on Lysine-Sepharose as we have previously described (Potempa and Nguyen, 2007, Skottrup *et al.*, 2011).

Gingipain activity against TIMP-1 and MMP-9

TIMP-1 or MMP-9 (400 μ g/ml, final concentration) was incubated with Kgp, RgpB or HRgpA (10 or 100 nM active protease) in 0.1 M Tris buffer pH 7.6, 2.5 mM CaCl₂, 75 mM NaCl, 0.01% NaN₃ supplemented with 10 mM L-cysteine at 37°C for 3 hours. The reactions were terminated by the addition of TLCK to 4 mM final concentration. The kinetics of TIMP-1 and MMP-9 degradation by gingipains were measured over 180 min, with reactions stopped at the appropriate time points with TLCK. Time-course analyses of TIMP-1 and MMP-9 hydrolysis were performed as above (10 nM active gingipain for MMP-9; 20 nM active gingipain for TIMP-1). Samples were boiled in reducing SDS-PAGE sample buffer, resolved by gradient SDS-PAGE (4-12 % NuPAGE Novex Bis-Tris minigels) and visualized with Sypro Ruby protein stain and a FluorChem HD2 image system (Alpha Innotech, Santa Clara, CA). Molecular masses were determined by comparison to the migration of Precision Plus pre-stained protein standards.

Statistical analyses

All data is presented as the mean $\pm$ standard deviation of triplicate experiments, unless otherwise stated. Differences between cytokine, MMP and MMP modulators produced in the gingival crevice model in the presence or absence of CSE were assessed by ANOVA. Differences in gingipains production in the presence and absence of CSE were assessed by t-test. Analyses were performed, and data presented, using Prism v5.02 and InStat v3.06 (Graphpad Software Inc., La Jolla, CA).

Results

Induction of cytokines by *P. gingivalis* in a gingival crevice model

Human gingival epithelial cells were stimulated with *P. gingivalis* in the presence or absence of primary human neutrophils and CSE. As is shown in Figure 1, cytokine production varied dramatically depending on the constituents of the crevicular crevice model. The presence of all three components (HGEC-Neutrophil-*P. gingivalis*) was required for meaningful detection of IL-1 β and IL-8, while CSE exposure significantly dampened pro-inflammatory cytokine production.

Induction of MMPs and MMP modulators by *P. gingivalis* in a gingival crevice model

As presented in Figure 2, while both neutrophils and epithelial cells are sources of large concentrations of MMP-9, neutrophils are the sole source of MMP-8 in the gingival crevice model. Levels of epithelial cell-derived MMP-9 are reduced in the presence of *P. gingivalis*, presumed to be due to proteolytic degradation, but significantly increased when HGEC, neutrophils and *P. gingivalis* are all present.

Neutrophils produced striking levels of the MMP-stabilizing molecule, NGAL, as shown in Figure 3. Epithelial cells produced significant amounts of the MMP-inhibitor, TIMP-1. Levels of TIMP-1 are reduced in the presence of *P. gingivalis*, again, presumed to be, due to proteolytic degradation. Neutrophils do not produce TIMP-1 (Opdenakker *et al.*, 2001). Therefore, neutrophils were able to partially protect epithelial-derived TIMP-1 in the crevicular crevice model. Interestingly, CSE further protected secreted epithelial cell-derived TIMP-1 in a dose-dependent manner.

CSE induces *P. gingivalis* gingipain production

As gingipains are key virulence factors of *P. gingivalis* that can promote host tissue destruction as well as degrading multiple mediators of the innate and adaptive immune responses, we next set out to establish if CSE influenced gingipain surface expression or secretion in two of the most commonly employed laboratory strains, ATCC 33277 and W83. CSE exposure suppressed cell-bound Kgp and Rgp gingipain production in *P. gingivalis* ATCC 33277, as shown in Figures 4A and C. On the other hand, CSE augmented cell-bound, Kgp and Rgp gingipain production in *P. gingivalis* W83 (Figure 4B). The effect of CSE exposure on the concentration of cell-free gingipains was not so impressive.

Gingipains degrade TIMP-1 and MMP-9

In gingival crevicular fluid collected from sites colonized with *P. gingivalis* gingipains are present at concentrations up to 100 nM (Guentsch *et al.*, 2011) and may affect balance of MMP and MMP-modulators in the gingival crevice and periodontal pockets. Therefore, to assess if gingipains can degrade MMP-9 and TIMP-1 we have incubated these proteins with RgpA, HRgpA and Kgp. As shown in Figure 5A TIMP-1 was totally degraded without any discrete cleavage products by Kgp and HRgpA applied at 10nM and 100nM concentrations. RgpB was somehow less effective in TIMP-1 digestion than other gingipains and after 3h

incubation with 10 nM RgpB some intact inhibitor was still observed in the gel (Figures 5 A and C). Also recombinant MMP-9 (rMMP-9), which occurred predominantly in the 92 kDa latent form with a smaller amount of an 82kDa isoform, assumed to be active MMP-9 was totally degraded by any gingipain at 100 nM concentration. At this gingipain concentration only the protease-derived bands were observed in gels after 3 h incubation (Figures 5 A and B). Conversely to TIMP-1 digestion, incubation of rMMP-9 with gingipains at 10 nM concentration released a discrete metalloprotease-derived cleavage products clearly visible after 3h. At this concentration of gingipains cleaved rMMP-9 in time-dependent manner releasing several discrete products (**Figure 5D**). Together this result argues that *in vivo* balance of MMP and MMP-modulators can be efficiently altered by bacteria-derived proteases.

Discussion

We have recently published some of the first data describing combinatorial interactions between the histopathologically dominant components of the diseased periodontium - bacteria (*P. gingivalis*), neutrophils, and epithelial cells (Galicía et al., 2009). Here we show that, while HGEs respond to oral bacteria in a manner that is considered critical in initiating the immune response, the introduction of neutrophils dramatically alters the dynamics of inflammation, as reflected in a surge in the production of cytokine and endogenous proteolysis-related factors. This likely occurs primarily due to both the release of inflammatory mediators from neutrophils but, perhaps, also due to the stabilizing effect of neutrophils on gingipain-exposed HGEs (Galicía et al., 2009).

MMP-8 and MMP-9 have both been reported to reflect the inflammatory burden in periodontal tissues and, thus, to represent biomarkers of periodontal disease (Leppilähti *et al.*, 2011, Ramseier *et al.*, 2009). Gingival crevicular modeling has permitted a number of novel insights into perturbations of the protease-antiprotease balance. Detection of total MMP-8 in supernatants of unstimulated or *P. gingivalis*-exposed HGEs was minimal. On the introduction of neutrophils, however, MMP-8 was abundant. MMP-8 production was increased and decreased at low and high CSE doses, respectively, whereas MMP-9 production was not influenced by exposure to CSE. Interestingly, *P. gingivalis* reduced the burden of HGE-derived MMP-9. Gingipains, which represent the bulk of the extensive proteolytic activity ascribed to *P. gingivalis*, are known to degrade multiple host proteins, including transferrin, defensins, complement proteins and cytokines (Guo *et al.*, 2010, Mezyk-Kopeck *et al.*, 2005). Recently, *P. gingivalis* has been suggested to activate MMP-9 by undefined mechanisms (Zhou *et al.*, 2011), while others have shown that dental plaque taken from periodontitis patients can convert MMP-9 into lower molecular mass forms (Ding *et al.*, 1995). We show, for the first time, that Kgp, RgpA and RgpB are each capable of the rapid and effective degradation of MMP-9. It should be noted that the considerable total MMP-9 released by neutrophils in the gingival crevicular model increases the overall MMP-9 burden, suggesting that the MMP-9-degrading capacity of the gingipains is saturated or blocked.

NGAL is a neutrophil granule protein that covalently interacts with and, subsequently, stabilizes MMP-9 (Yan *et al.*, 2001). We report the novel finding that NGAL was released by neutrophils at impressive levels in the crevicular model. As there are some reports of epithelial-derived NGAL, the source of NGAL in mucosal inflammatory diseases has been debated (Eagan *et al.*, 2010). In our tripartite model, neutrophils are clearly the major contributors to *P. gingivalis*-induced NGAL and the possibility of significant gingipain-mediated NGAL degradation was not suggested by the data. This neutrophil-derived NGAL would be expected to prolong MMP-9 activity, augmenting to the collagenolytic activity that is a hallmark of periodontitis. As well as prolonging MMP-9 activity, NGAL takes part in

the anti-bacterial iron depletion strategy of the innate immune system through its ability to tightly bind to siderophores of the catechol type (Goetz *et al.*, 2002) and NGAL may also protect the host against reactive oxygen species (Roudkenar *et al.*, 2008).

TIMP-1, the major endogenous MMP inhibitor, is thought not to be produced by neutrophils (Opdenakker *et al.*, 2001). HGECs, therefore, unsurprisingly represented the sole source of TIMP-1 in the current system. We have shown for the first time that, similarly to MMP-9, *P. gingivalis* abrogated HGEC-derived TIMP-1, and the addition of neutrophils was able to only partially restore TIMP-1 concentrations. Therefore, we went on to establish that Kgp, RgpA and RgpB are each capable of the rapid and effective degradation of TIMP-1. Again, TIMP-1 degradation would be expected to augment MMP-mediated collagenolytic activity and abet the progression of periodontitis. These data are in keeping with a prior report that whole *P. gingivalis* can degrade TIMP-1 by an unidentified mechanism (Grenier and Mayrand, 2001).

W83 (CSE upregulates gingipain production) is a high virulence strain, relative to 33277 (Lin *et al.*, 2009, Naito *et al.*, 2008). W83 and 33277 both express Kgp, HRgpA and HRgpB. While the C-terminal hemagglutinin /adhesion 3/4 domain junction differs between the Kgp of W83 and 33277, this protein region is non-catalytic and not involved in gingipain processing or transport (Li *et al.*, 2010, Sztukowska *et al.*, 2004) and, thus, is unlikely to explain the differential gingipain response to CSE. Thus, the mechanisms underlying strain-specific variation in cell-associated gingipain activity upon CSE exposure remain unclear.

Concentrations of IL-8 and IL-1 β in gingival crevicular fluid are known to correlate with periodontal disease severity (Ertugrul *et al.*, 2012). Multiple oral bacteria, in both planktonic and biofilm form, are known to induce IL-1 β and IL-8, and other pro-inflammatory cytokines, from human oral epithelial cells (Peyyala *et al.*, 2012). Neutrophils are also known to secrete large amounts of IL-8 and IL-1 β (Cassatella, 1995). IL-1 and IL-8 levels detected in 24 hour HGEC supernatants upon stimulation with *P. gingivalis* alone were low, but similar to our prior report using live *P. gingivalis* (Stathopoulou *et al.*, 2009). Significantly higher cytokine levels were found on HGEC stimulation with dead *P. gingivalis* or with gingipain deficient mutants (Stathopoulou *et al.*, 2009). In the tripartite gingival crevice model, it appears that neutrophils are the primary source IL-8 and IL-1 β , although it cannot be ruled out that neutrophils induce IL-8 and IL-1 β from epithelial cells in a manner that *P. gingivalis* alone does not. Alternatively, *P. gingivalis*-derived proteases may be sufficient to degrade both IL-1 β and IL-8 produced by *P. gingivalis*-stimulated epithelial cells but not by the combination of neutrophils and epithelial cells.

Several studies have shown that pro-inflammatory cytokines, including IL-1 β and IL-8, are reduced in the gingival crevicular fluid of smokers with periodontal diseases compared to diseased non-smokers (Tymkiw *et al.*, 2011, Rawlinson *et al.*, 2003, Palmer *et al.*, 2005). Mechanistically, it is known that smoke exposure alters the activity of multiple *P. gingivalis* genes and the expression levels of several membrane proteins that result in a lower inflammatory potential of this key pathogen (Bagaitkar *et al.*, 2011, Bagaitkar *et al.*, 2010, Bagaitkar *et al.*, 2009). Similarly, it is known that the result of nicotine, and the major human nicotine metabolite, cotinine, engagement of innate cell $\alpha 7$ -nicotinic acetylcholine receptors is the activation of the cholinergic anti-inflammatory and augmentation of the endogenous Akt / GSK-3 β anti-inflammatory pathways (Rehani *et al.*, 2008, Martin *et al.*, 2005, Tracey, 2007). Thus, cytokine profiles in the tripartite model are in keeping with established clinical and mechanistic phenomena. Furthermore, the reduced inflammatory response to *P. gingivalis* upon CSE exposure is likely to promote *P. gingivalis* survival. Again, this is in keeping with multiple studies have shown that, relative to non-smokers, smokers are more likely to be infected with *P. gingivalis*, to be infected with higher numbers

of this bacterium, and to exhibit more persistent infection by this pathogen (Haffajee and Socransky, 2001, Zambon *et al.*, 1996, Eggert *et al.*, 2001, Kamma *et al.*, 1999). Importantly, then, exploitation of crevicular modeling will facilitate a deeper mechanistic understanding of the immune suppression that occurs in the periodontia of smokers and may well have relevance to the general increase in susceptibility to bacterial infections in tobacco users (Bagaitkar *et al.*, 2008).

While MMPs are known to contribute to the destruction of periodontal tissues in smokers and non-smokers alike (Leppilahti *et al.*, 2011, Ramseier *et al.*, 2009), the balance between MMPs and endogenous mediators has consistently been shown to be altered in cigarette smokers (Gursoy *et al.*, 2010, Heikkinen *et al.*, 2010, Liede *et al.*, 1999). Recently, Mouzakiti *et al.* have shown that TIMP-1 mRNA levels in human periodontal tissues removed from smoker subjects with untreated chronic periodontitis are higher in smokers than in non-smokers (Mouzakiti *et al.*, 2011). Several studies have reported reduced MMP-8 concentrations in oral fluids of smokers with periodontitis compared to non-smoker controls (Heikkinen *et al.*, 2011, Heikkinen *et al.*, 2010, Liede *et al.*, 1999, Mantyla *et al.*, 2006), although tissue levels of MMP-8 may be increased (Liu *et al.*, 2006). Thus, the data generated by our *in vitro* model of the gingival crevice reflect the reported *in vivo* data, strengthening the suitability of our model for the mechanistic dissection of innate-pathogen interactions in periodontal diseases.

The influence of tobacco smoke exposure on gingipains production has, to the best of our knowledge, not been previously addressed. We show that cell-bound Rgp and Kgp are decreased in CSE-treated *P. gingivalis* ATCC 33277, the type strain, relative to control bacteria, whereas both Rgp and Kgp are significantly upregulated in the highly virulent *P. gingivalis* W83 strain. Thus, the influence of tobacco smoke on gingipain production may be strain-specific.

While the potential for variation in results produced by this model due to genetic differences in primary cell isolates must be acknowledged, it will be possible to incorporate primary cells phenotyped for key innate receptors, such as TLR polymorphisms (Kinane *et al.*, 2006).

In summary, the current findings underscore the importance of neutrophils in modifying the responses of primary gingival epithelial cells to bacterial insults. Rgp and Kgp gingipains are capable of degrading MMP-9 and TIMP-1. Furthermore, CSE-exposure exerts multiple influences on the gingival crevice model consistent with the promotion of *P. gingivalis* infection and established mechanistic phenomena and clinical observations. Thus, the potential utility of the gingival crevice model for mechanistic studies at the cellular and molecular levels is established. Exploitation of the model may help identify therapeutic targets for the treatment of tobacco-induced periodontitis and other chronic inflammatory mucosal diseases. Indeed, this initial publication has already identified several novel mechanisms likely to contribute to periodontal disease progression including *P. gingivalis*-induced NGAL secretion, *P. gingivalis*-induced, gingipain-mediated MMP-9 and TIMP-1 degradation, and CSE-induced alterations to gingipain production.

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Figure 1. CSE suppresses the production of pro-inflammatory cytokines in a gingival crevice model

Human gingival epithelial cells were stimulated with *P. gingivalis* in the presence or absence of primary human neutrophils and CSE. HGEc: *P. gingivalis*; neutrophil ratio was 1:100:1. Concentrations of (A) IL-1 β and (B) IL-8 in 24 hr cell-free supernatants of *P. gingivalis*-

stimulated HGECs in the presence or absence of neutrophils with or without CSE was measured by ELISA.

HGEC only (dotted bars); HGEC plus *P. gingivalis* (checked bars); HGEC plus *P. gingivalis* plus neutrophils (white bars); HGEC plus *P. gingivalis* plus neutrophils plus nicotine at 100 ng / ml nicotine equivalency (grey bars); HGEC plus *P. gingivalis* plus neutrophils plus nicotine at 100 ng / ml nicotine equivalency (blackbars).

*** $p < 0.001$; ** $p < 0.01$.

Figure 2. Burden of total MMP-8 and MMP-9 in a gingival crevice model

Human gingival epithelial cells were stimulated with *P. gingivalis* in the presence or absence of primary human neutrophils and CSE. HGEc: *P. gingivalis*; neutrophil ratio was 1:100:1. Concentrations of (A) MMP-8 and (B) MMP-9 in 24 hr cell-free supernatants of *P.*

gingivalis-stimulated HGECs in the presence or absence of neutrophils with or without CSE was measured by ELISA. HGEC only (dotted bars); HGEC plus *P. gingivalis* (checked bars); HGEC plus *P. gingivalis* plus neutrophils (white bars); HGEC plus *P. gingivalis* plus neutrophils plus nicotine at 100 ng / ml nicotine equivalency (grey bars); HGEC plus *P. gingivalis* plus neutrophils plus nicotine at 100 ng / ml nicotine equivalency (blackbars). *** $p < 0.001$; ** $p < 0.01$; $p < 0.05$.

Figure 3. Production of MMP-modulators in a gingival crevice model

Human gingival epithelial cells were stimulated with *P. gingivalis* in the presence or absence of primary human neutrophils and CSE. HGEC: *P. gingivalis*: neutrophil ratio was 1:100:1. Concentrations of (A) NGAL and (B) TIMP-1 in 24 hr cell-free supernatants of *P. gingivalis*-stimulated HGECs in the presence or absence of neutrophils and CSE was measured by ELISA. HGEC only (dotted bars); HGEC plus *P. gingivalis* (checked bars);

HGEC plus *P. gingivalis* plus neutrophils (white bars); HGEC plus *P. gingivalis* plus neutrophils plus nicotine at 100 ng / ml nicotine equivalency (grey bars); HGEC plus *P. gingivalis* plus neutrophils plus nicotine at 100 ng / ml nicotine equivalency (blackbars).
*** $p < 0.001$; ** $p < 0.01$; $p < 0.05$.

Figure 4. Influence of CSE on gingipain production is *P. gingivalis* strain-specific

The influence of CSE on gingipain production by two strains of *P. gingivalis* was assessed. Total Kgp activity in control and CSE-exposed cultures of (A) *P. gingivalis* 33277 and (B) *P. gingivalis* W83 was determined by spectrophotometric analysis of acetyl-lysine-*p*-nitroanilide degradation. Total Rgp activity in control and CSE-exposed cultures of (C) *P. gingivalis* 33277 and (D) *P. gingivalis* W83 was determined by spectrophotometric analysis of *N*- α -benzoyl-D,L-arginine-*p*-nitroanilide degradation. $p < 0.001$; ** $p < 0.01$; $p < 0.05$.

Figure 5. *P. gingivalis* gingipains degrade TIMP-1 and MMP-9

The ability of RgpA, HRgpA and Kgp gingipains to degrade TIMP-1 and MMP-9 was assessed. Recombinant TIMP-1 (A) or MMP-9 (B), 400 μ g/ml, were incubated with RgpA, HRgpA or Kgp (active proteases 10 and 100 nM) at 37°C in gingipain activity buffer with 10 mM L-cysteine. After 3 hours, TLCK was added to block the reaction and residual proteins were visualized by SDS-PAGE under reducing conditions. The degradation of TIMP-1 (C) and MMP-9 (D) over 180 min was also monitored. TIMP-1 and MMP-9 incubated without gingipains served as negative controls. Typical digests are presented.