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

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NEUTROPHILS ALTER EPITHELIAL RESPONSE TO *PORPHYROMONAS*
GINGIVALIS IN A GINGIVAL CREVICE MODEL

By

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B.S., University of Louisville, 2010

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A Thesis Approved on

April 21, 2014

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ABSTRACT

NEUTROPHILS ALTER EPITHELIAL RESPONSE TO *PORPHYROMONAS GINGIVALIS* IN A GINGIVAL CREVICE MODEL

Jessica Lynn Bondy-Carey

April 21, 2014

A gingival crevice model (epithelial cell–*Porphyromonas gingivalis*–neutrophil) was established and used to profile gingipain, matrix metalloproteinase (MMP), MMP mediators [neutrophil gelatinase-associated lipocalin (NGAL) and tissue inhibitor of metalloproteinases 1 (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.

Porphyromonas gingivalis alone induced low levels of interleukin-1b and interleukin-8 from epithelial cells, but high levels of both cytokines were produced on the addition of neutrophils. Exposure to CSE (100 and 1000 ng mL⁻¹ nicotine equivalency) significantly compromised *P. gingivalis*-induced cytokine secretion (both $P < 0.05$). *P. gingivalis* induced impressive secretion of NGAL ($P < 0.05$) that was not influenced by CSE. The influence of CSE on gingipain 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. Hence, *P. gingivalis* exerts a complex influence on the proteolytic balance of a gingival crevice model. Exposure to CSE reduces the proinflammatory 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. This straightforward gingival crevice model is established as a suitable vehicle for the elucidation of mechanisms that contribute to susceptibility to periodontitis.

TABLE OF CONTENTS

	PAGE
ACKNOWLEDGEMENTS.....	iii
ABSTRACT.....	iv
LIST OF FIGURES.....	vii
INTRODUCTION.....	1
METHODS AND MATERIALS.....	4
Materials.....	4
Bacterial culture.....	5
Gingival epithelial cell isolation and culture.....	5
Conditioning of growth media.....	6
Isolation of primary human neutrophils.....	6
Epithelial–neutrophil– <i>P. gingivalis</i> interactions.....	6
Cytokine profiling.....	7
Profiling of MMPs and regulators.....	7
Gingipain profiling.....	7
Purification of Rgp and Kgp gingipains.....	8
Gingipain activity against TIMP-1 and MMP-9.....	8
Statistical analyses.....	9
RESULTS.....	10
Induction of cytokines by <i>P. gingivalis</i> in a gingival crevice model.....	10

	PAGE
Induction of MMPs and MMP modulators by <i>P. gingivalis</i> in a gingival crevice model.....	10
CSE induces <i>P. gingivalis</i> gingipain production.....	13
Gingipains degrade TIMP-1 and MMP-9.....	13
DISCUSSION.....	17
REFERENCES.....	24
APPENDICES.....	30
CURRICULUM VITA.....	31

LIST OF FIGURES

FIGURE	PAGE
1. Figure 1.....	11
2. Figure 2.....	11
3. Figure 3.....	12
4. Figure 4.....	15
5. Figure 5.....	16

INTRODUCTION

Periodontitis is a common infectious, chronic inflammatory disease of the supportive structures of the teeth (Tonetti, 2009; Detert et al., 2010). Furthermore, multiple serious systemic sequelae are associated with the establishment of destructive periodontal lesions, including vascular and pulmonary diseases, preterm birth, rheumatoid arthritis and diabetes complications (Scannapieco et al., 2010). Histopathological studies and clinical assays have established that, although 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 (Galicia et al., 2009; Scott & Krauss, 2011). Despite this, most studies of *Porphyromonas gingivalis*–host interactions have included epithelial cells or leukocytes, but not both, our own work included (Sandros et al., 2000; Rehani et al., 2008; Bagaitkar et al., 2009).

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 (HGEC) 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 a result of efficient phagocytosis and bacterial killing by neutrophils. Furthermore, bacteria-induced cytokine production, particularly of interleukin-1 β (IL-1 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 USA (Tomar & 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 nonsmokers (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), is likely to contribute to the progression of periodontitis in smokers. Of the endogenous proteases, matrix metalloproteinases (MMPs) – the therapeutic target of subantimicrobial doses of tetracyclines – are considered of prime importance (Gu et al., 2011).

Therefore, we assessed, in a tripartite gingival crevice model, (i) the production of representative proinflammatory cytokines (IL-1 β and IL-8); MMPs

(MMP-8 and MMP-9); and MMP-modulators [tissue inhibitor of metalloproteinases 1 (TIMP-1) and neutrophil gelatinase-associated lipocalin (NGAL)] and (ii) the effect of cigarette smoke extract (CSE) on these key mediators of inflammation; and hence we attempted to establish the relevance of a crevicular model with respect to established in vivo phenomena, to permit future mechanistic studies. The influence of CSE on *P. gingivalis*-derived gingipains was also assessed.

METHODS

Materials

P. gingivalis strains W83, HG66 and ATCC 33277 were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Unless otherwise stated, ATCC 33277 was used in all experiments. Gifu Anaerobe Medium (GAM) came from Nissui Pharmaceutical (Tokyo, Japan). 2-Aminoethanol, amphotericin B (fungizone), insulin, 2-mercaptoethanol, penicillin/streptomycin, Sephadex G 150, sodium selenite, transferrin and sodium-*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). The IL-1 β and IL-8 enzyme-linked immunosorbent assay (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; Kgp: acetyl-lysine-*p*-nitroanilide) were purchased from Sigma and Bachem America, Inc. (Torrance, CA), respectively.

Bacterial culture

P. gingivalis was grown in 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 phosphate buffered saline (pH 7.4), and used immediately.

Gingival epithelial cell isolation and culture

Primary HGEC were isolated from trypsinated, periodontally healthy gingival tissue biopsies obtained from patients undergoing crown-lengthening procedures, as previously described (Guggenheim et al., 2009). Informed and written consent was obtained from tissue donors, as approved by the University of Louisville, Institutional Review Board, Human Subjects Protection Program (Study # 619-03). The HGEC were seeded in 60-mm plastic tissue culture plates coated with type-I collagen and incubated in 5% CO₂ at 37°C using KSFM containing 10 $\mu\text{g ml}^{-1}$ insulin, 5 $\mu\text{g ml}^{-1}$ transferrin, 10 μM 2-mercaptoethanol, 10 μM 2-aminoethanol, 10 mM sodium selenite, 50 $\mu\text{g ml}^{-1}$ bovine pituitary extract, 100 units ml^{-1} penicillin/streptomycin and 50 ng ml^{-1} fungizone (complete

medium). Antibiotic-free medium was used in all experiments involving the use of bacteria.

Conditioning of growth media

The 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 s, as previously described (Bagaitkar et al., 2009). Media were sterile filtered (0.22 μm); diluted to the required concentration in nicotine equivalents (0, 100 and 1000 ng ml^{-1}), 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 anticoagulated 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

The HGEC–neutrophil–*P. gingivalis* interactions were progressed as previously described (Galicia et al., 2009). Monolayers of HGEC (1×10^6 cells; fourth passage) were overlaid with viable neutrophils (1×10^6 cells) and placed back in the incubator for 30 min. Live *P. gingivalis* (1×10^8 cells) in KSFM or KSFM supplemented with CSE (100 and 1000 ng ml⁻¹ nicotine equivalents) were added to the HGEC–neutrophil coculture for 24 h.

Cytokine profiling

Cytokine (IL-1 β and IL-8) concentrations in 24-h 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 MMPs and regulators

Concentrations of MMP-8, MMP-9, NGAL and TIMP-1 in 24-h 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⁻¹) using sterile media. Cell-free supernatants were removed and corresponding cell pellets were re-suspended in buffer supporting

gingipain activity (0.2 M Tris–HCl, 0.1 M NaCl, 5 mM 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 or acetyl-lysine-*p*-nitroanilide at 1 mM final concentration with absorbance measured at 405 nm after 3 h, as we have previously described (Potempa & 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 cell-free medium of *P. gingivalis* W83, by acetone precipitation, size exclusion chromatography using Sephadex G-150, and affinity chromatography on Lysine–Sepharose as previously described (Potempa & Nguyen, 2007; Skottrup et al., 2011).

Gingipain activity against TIMP-1 and MMP-9

TIMP-1 or MMP-9 (400 $\mu\text{g ml}^{-1}$, final concentration) was incubated with Kgp, RgpB or HRgpA (10 or 100 nM active protease) in 0.1 M Tris–HCl 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 h. 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 described above (10 nM active gingipain for MMP-

9; 20 nM active gingipain for TIMP-1). Samples were boiled in reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (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 prestained protein standards.

Statistical analyses

All data are 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 analysis of variance. Differences in gingipain production in the presence and absence of CSE were assessed by *t*-test. Analyses were performed, and data are 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 Fig. 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, whereas CSE exposure significantly dampened proinflammatory cytokine production.

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

As presented in Fig. 2, although 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*, presumably as a result of proteolytic degradation, but are significantly increased when HGEC, neutrophils and *P. gingivalis* are all present. Neutrophils produced striking levels of the MMP stabilizing molecule, NGAL, as shown in Fig. 3. Epithelial cells produced significant amounts of the MMP inhibitor, TIMP-1. Levels of TIMP-1 are reduced

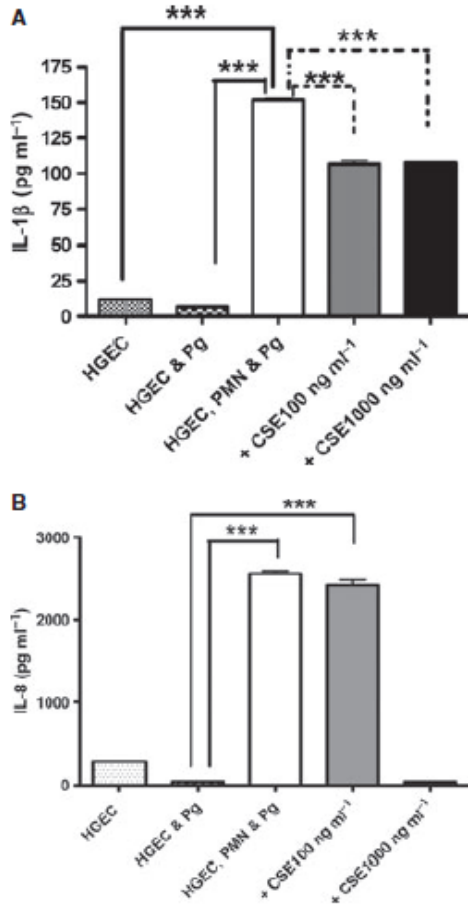


Figure 1: Cigarette smoke extract (CSE) suppresses the production of proinflammatory cytokines in a gingival crevice model. Human gingival epithelial cells (HGEC) were stimulated with *Porphyromonas gingivalis* in the presence or absence of primary human neutrophils and CSE. The HGEC : *P. gingivalis* : neutrophil ratio was 1 : 100 : 1. Concentrations of (A) interleukin-1 β (IL-1 β) and (B) IL-8 in 24-h cell-free supernatants of *P. gingivalis* stimulated HGECs in the presence or absence of neutrophils with or without CSE was measured by enzyme-linked immunosorbent assay. 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⁻¹ nicotine equivalency (gray bars); HGEC plus *P. gingivalis* plus neutrophils plus nicotine at 1000 ng ml⁻¹ nicotine equivalency (black bars). ***P < 0.001; **P < 0.01.

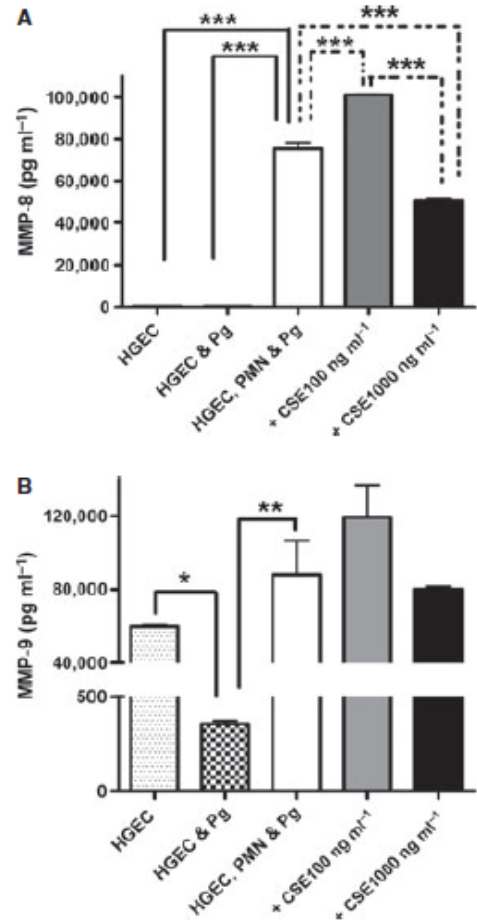
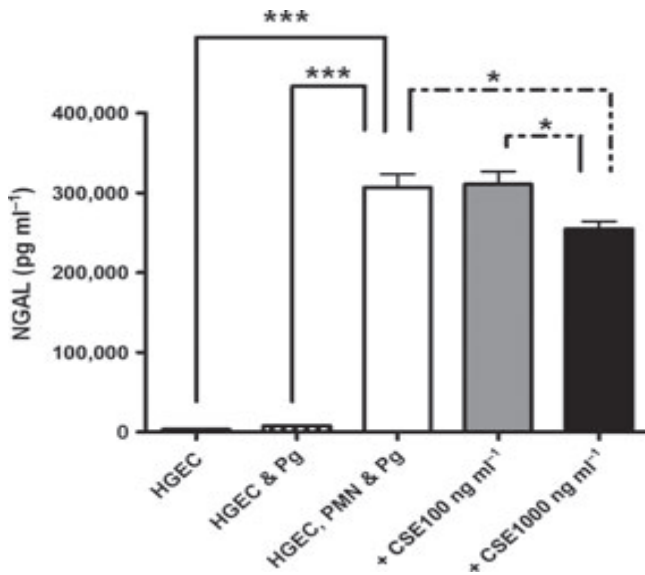


Figure 2: Burden of total matrix metalloproteinase 8 (MMP-8) and MMP-9 in a gingival crevice model. Human gingival epithelial cells (HGEC) were stimulated with *Porphyromonas gingivalis* in the presence or absence of primary human neutrophils and cigarette smoke extract (CSE). The HGEC : *P. gingivalis* : neutrophil ratio was 1 : 100 : 1. Concentrations of (A) MMP-8 and (B) MMP-9 in 24-h cell-free supernatants of *P. gingivalis*-stimulated HGECs in the presence or absence of neutrophils with or without CSE was measured by enzyme-linked immunosorbent assay. 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⁻¹ nicotine equivalency (gray bars); HGEC plus *P. gingivalis* plus neutrophils plus nicotine at 1000 ng ml⁻¹ nicotine equivalency (black bars). ***P < 0.001; **P < 0.01; *P < 0.05.

A



B

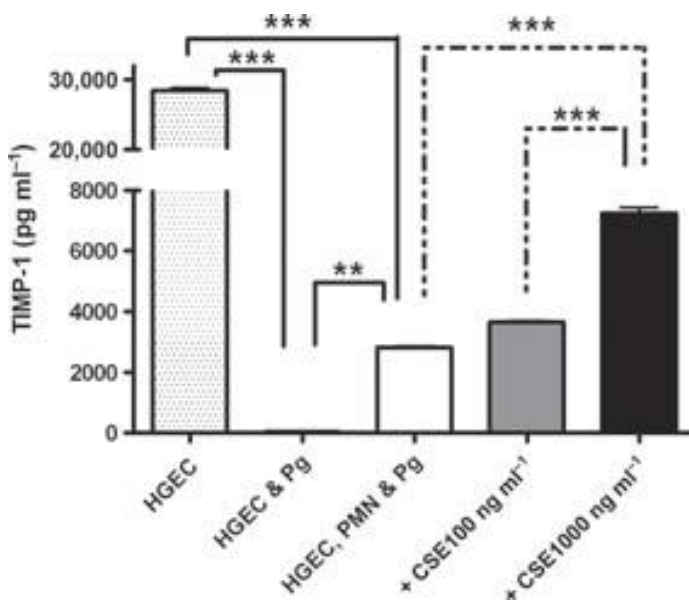


Figure 3: Production of matrix metalloproteinase (MMP) modulators in a gingival crevice model. Human gingival epithelial cells (HGEC) were stimulated with *Porphyromonas gingivalis* in the presence or absence of primary human neutrophils and cigarette smoke extract (CSE). The HGEC : *P. gingivalis* : neutrophil ratio was 1 : 100 : 1. Concentrations of (A) neutrophil gelatinase-associated lipocalin (NGAL) and (B) tissue inhibitor of metalloproteinases 1 (TIMP-1) in 24-h cell-free supernatants of *P. gingivalis*-stimulated HGECs in the presence or absence of neutrophils and CSE was measured by enzyme-linked immunosorbent assay. 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⁻¹ nicotine equivalency (gray bars); HGEC plus *P. gingivalis* plus neutrophils plus nicotine at 1000 ng ml⁻¹ nicotine equivalency (black bars). ***P < 0.001; **P < 0.01; *P < 0.05

in the presence of *P. gingivalis*, again presumably as a result of 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. Exposure to CSE suppressed cell-bound Kgp and Rgp gingipain production in *P. gingivalis* ATCC 33277, as shown in Fig. 4A,C. On the other hand, CSE augmented cell-bound, Kgp and Rgp gingipain production in *P. gingivalis* W83 (Fig. 4B). The effect of CSE exposure on the concentration of cell-free gingipains was not so remarkable.

Gingipains degrade TIMP-1 and MMP-9

In gingival crevicular fluid collected from sites colonized by *P. gingivalis* gingipains are present at concentrations up to 100 nM (Guentsch et al., 2011) and may affect the 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 Fig. 5A TIMP-1 was totally degraded without any discrete cleavage products by Kgp and HRgpA applied at 10 and 100 nM concentrations. RgpB was somehow less effective in TIMP-1 digestion than other gingipains and after 3 h of incubation with 10 nM RgpB some intact inhibitor was still observed in the gel (Fig. 5A,C). Also, recombinant MMP-9 (rMMP-9), which occurred predominantly in the 92-kDa latent form with a smaller amount of an 82-kDa 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 of incubation (Fig. 5 A,B). Conversely to TIMP-1 digestion, incubation of rMMP-9 with gingipains at 10 nM concentration released discrete metalloprotease-derived cleavage products clearly visible after 3 h. At this concentration of gingipains, rMMP-9 was cleaved in a time-dependent manner, releasing several discrete products (Fig. 5D). Together this result argues that the *in vivo* balance of MMP and MMP-modulators may be efficiently altered by bacteria-derived proteases.

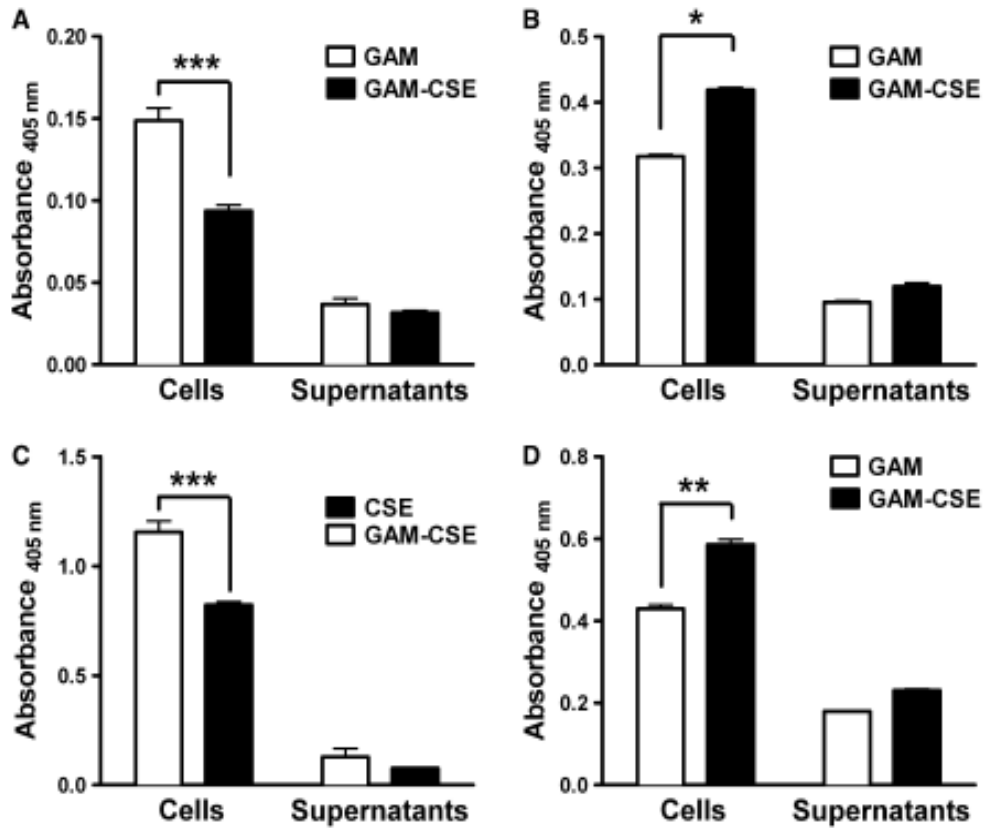


Figure 4: Influence of cigarette smoke extract (CSE) on gingipain production is *Porphyromonas gingivalis* strain-specific. The influence of CSE on gingipain production by two strains of *P. gingivalis* was assessed. Total Kgp activity in control and CSE-exposed cultures of (A) *P. gingivalis* 33277 and (B) *P. gingivalis* W83 was determined by spectrophotometric analysis of acetyl-lysine-*p*-nitroanilide hydrolysis. 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-DL-arginine-*p*-nitroanilide hydrolysis. ***P < 0.001; **P < 0.01; *P < 0.05.

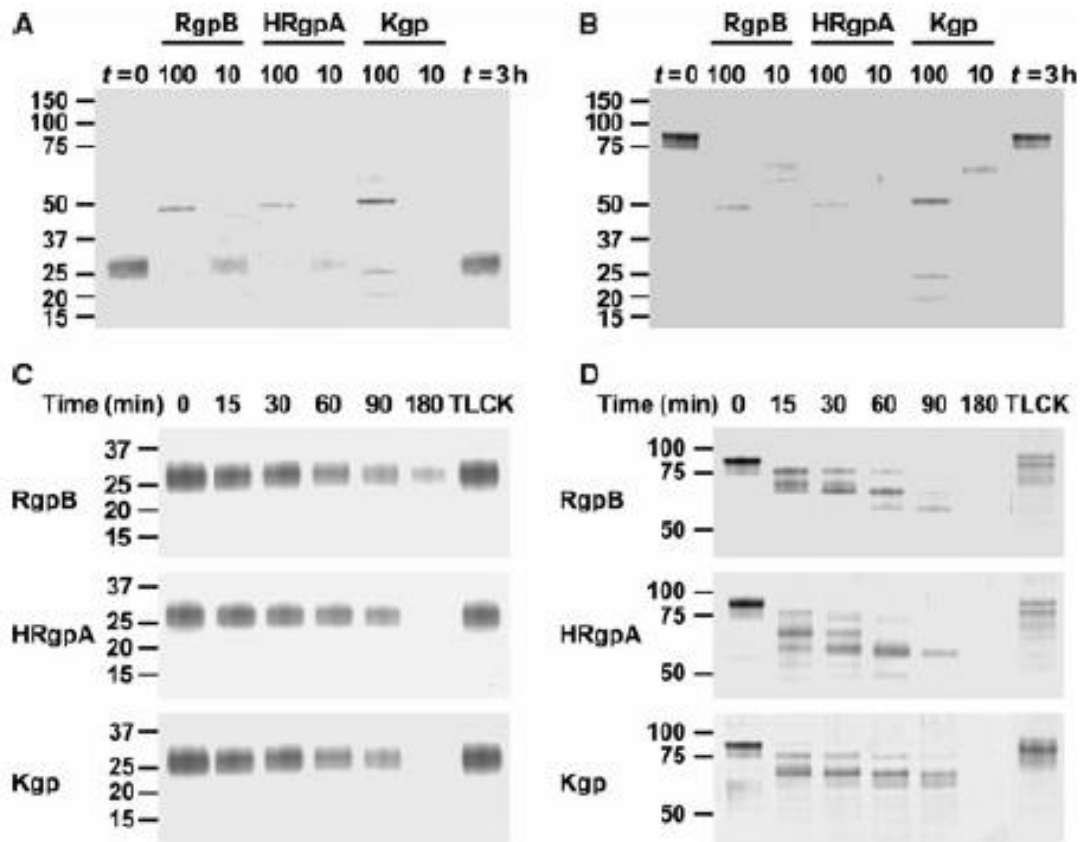


Figure 5: *Porphyromonas gingivalis* gingipains degrade tissue inhibitor of metalloproteinases 1 (TIMP-1) and matrix metalloproteinase 9 (MMP-9). The ability of RgpA, HRgpA and Kgp gingipains to degrade TIMP-1 and MMP-9 was assessed. Recombinant TIMP-1 (A) or MMP-9 (B), $400 \mu\text{g ml}^{-1}$, were 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 h, sodium-*p*-tosyl-L-lysine chloromethyl ketone was added to block the reaction and residual proteins were visualized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis 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.

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, although HGEC 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 as a result of the release of inflammatory mediators from neutrophils but, perhaps, also through the stabilizing effect of neutrophils on gingipain-exposed HGEC (Galicía et al., 2009).

Both MMP-8 and MMP-9 have been reported to reflect the inflammatory burden in periodontal tissues and so they represent biomarkers of periodontal disease (Ramseier et al., 2009; Leppilähti et al., 2011). 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 HGEC 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 HGEC-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 (Mezyk-Kopec et al., 2005; Guo et al., 2010). Recently, *P. gingivalis* has been suggested to activate MMP-9 by undefined mechanisms (Zhou et al., 2012), whereas others have shown that dental plaque taken from patients with periodontitis 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 high levels in the crevicular model. As there are some reports of epithelially-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 the collagenolytic activity that is a hallmark of periodontitis. As well as prolonging MMP-9 activity, NGAL takes part in the antibacterial iron depletion strategy of the innate immune system

through its ability to tightly bind to siderophores of the catecholate 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). HGEC, 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 previous report that whole *P. gingivalis* can degrade TIMP-1 by an unidentified mechanism (Grenier & Mayrand, 2001).

W83 (in which CSE upregulates gingipain production) is a high virulence strain, relative to 33277 (Naito et al., 2008; Lin et al., 2009). Both W83 and 33277 express Kgp, HRgpA and HRgpB. Although 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 (Sztukowska et al., 2004; Li et al., 2010), so this is unlikely to explain the differential gingipain response to CSE. Hence, 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 proinflammatory 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). Levels of IL-1 β and IL-8 detected in 24-h HGEC supernatants upon stimulation with *P. gingivalis* alone were low, but similar to our previous 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 for 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 proinflammatory cytokines, including IL-1 β and IL-8, are reduced in the gingival crevicular fluid of smokers with periodontal diseases compared with diseased non-smokers (Rawlinson et al., 2003; Palmer et al., 2005; Tymkiw et al., 2011). 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., 2009, 2010, 2011).

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 (Martin et al., 2005; Tracey, 2007; Rehani et al., 2008). Hence, 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 showing 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 (Zambon et al., 1996; Kamma et al., 1999; Eggert et al., 2001; Haffajee & Socransky, 2001). Exploitation of crevicular modeling will facilitate a deeper mechanistic understanding of the immune suppression that occurs in the periodontium of smokers and may well have relevance for the general increase in susceptibility to bacterial infections in tobacco users (Bagaitkar et al., 2008).

Although MMPs are known to contribute to the destruction of periodontal tissues in smokers and non-smokers alike (Ramseier et al., 2009; Leppilahti et al., 2011), the balance between MMPs and endogenous mediators has consistently been shown to be altered in cigarette smokers (Liede et al., 1999; Gursoy et al., 2010; Heikkinen et al., 2010). Recently, Mouzakiti et al. (2011) have shown that TIMP-1 messenger RNA levels in human periodontal tissues removed from smokers with untreated chronic periodontitis are higher than in

non-smokers. Several studies have reported reduced MMP-8 concentrations in oral fluids of smokers with periodontitis compared with non-smoking controls (Liede et al., 1999; Mantyla et al., 2006; Heikkinen et al., 2010, 2012), although tissue levels of MMP-8 may be increased (Liu et al., 2006). Hence, 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 gingipain 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. The influence of tobacco smoke on gingipain production may be strain-specific.

The potential for variation in the results produced by this model caused by genetic differences in primary cell isolates must be acknowledged, but it will be possible to incorporate primary cells phenotyped for key innate receptors, such as Toll-like receptor 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. Hence, 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 to 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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APPENDIX 1

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APPENDIX 2
ABBREVIATIONS

CSE= cigarette smoke extract

ELISA= enzyme-linked immunosorbent assay

GAM= Gifu anaerobe medium

HGEC= human gingival epithelial cells

IL-1 β = interleukin-1 β

IL-8= interleukin-8

KSFM= keratinocyte serum-free medium

MMPs= matrix metalloproteinases

NGAL= neutrophil gelatinase-associate lipocalin

SDS-PAGE= sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TIMP-1= tissue inhibitor of metalloproteinases 1

TLCK= sodium-*p*-tosyl-L-lysine chloromethyl ketone

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