INTERACTIONS OF Porphyromonas gingivalis WITH HUMAN PLACENTAL CELLS AND THE IMPLICATIONS FOR PRETERM BIRTH

By

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To my family
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INTERACTIONS OF PORPHYROMONAS GINGIVALIS WITH HUMAN PLACENTAL CELLS AND THE IMPLICATIONS FOR PRETERM BIRTH

By

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Chair: Richard J. Lamont
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Objective: Preterm birth is the leading cause of infant morbidity and mortality and affects 12.5% of births in the United States. Epidemiological studies have indicated periodontal disease to be a risk factor for preterm birth. Mechanistically, the cause could be oral-hematogenous spread of Porphyromonas gingivalis to the gestational tissues inducing inflammation. Hence, the objective was to examine the interactions between P. gingivalis and placental tissues.

Methods: Human placental trophoblasts (HTR-8/SVneo cell line) were co-cultured with P. gingivalis (or a control of no bacteria) for 2 hours at 37°C at a multiplicity of infection of 200 bacteria per cell. For microarrays total RNA was extracted, purified, quantified, reverse transcribed, and used to probe Affymetrix HG-U133 Plus 2.0 human microarrays. Differential expression patterns were analyzed with bio-informatics, statistical, and gene ontology tools and databases.

Images of infected cells were taken with the confocal laser scanning microscope. Western blotting was used to analyze the phosphorylation state of factor involved the p38 MAPK pathway. ELISAs were performed on culture supernatants and lysates to verify cytokine expression.
**Results:** Among the pathways and factors up-regulated at the mRNA level by exposure to *P. gingivalis* were pathways for cytokine signaling. Confocal images showed attachment of *P. gingivalis* to the HTR-8/SVNeo cells. Western blots showed activation of the p38 MAPK pathway. ELISAs showed expression of cytokines IL-8 and IL-6 were increased after exposure to *P. gingivalis* relative to the control.

**Conclusions:** Exposure of HTR-8/SVneo human trophoblast cells to *P. gingivalis* leads to the up-regulation and increased production of inflammatory cytokines and induction of pathways leading to inflammation.
CHAPTER 1
INTRODUCTION

Preterm Birth

Preterm birth is defined as birth occurring before the 37th week of gestation. It is the leading cause of infant morbidity and mortality and affects 12.5% of births in the United States. Preterm babies are at increased risk of neurological, respiratory, and gastrointestinal problems (Goldenberg et al., 2008).

Preterm birth is either induced or spontaneous. Labor may be induced, or a caesarean section performed if it becomes necessary for the health of the mother or baby. However the majority of preterm births occur following spontaneous preterm labor with or without preterm premature rupture of the membranes. Spontaneous preterm labor has many causes with infection being present about 50% of the time (Michalowicz & Durand, 2007). Routes of uterine infection include ascending from the vagina and cervix through the genital tract, hematogenous dissemination from the maternal bloodstream through the placenta, retrograde seeding from the peritoneal cavity, and accidental introduction during invasive procedures (Romero et al., 2006).

The placenta functions as an interface between mother and fetus, allowing for the exchange of nutrients and waste products. The placenta is formed from the trophoblast layer of the blastocyst which forms 4 days after fertilization. The blastocyst is a thin-walled, hollow, fluid filled structure made up of four components. These components are a glycoprotein membrane, the trophoblast which forms the placenta, the embryoblast which will form the embryo, and a fluid filled cavity. (Blackburn, 2007)

A normal pregnancy lasts 40 weeks. Parturition can be divided into four phases, quiescence, activation of the fetal hypothalamic-pituitary adrenal (HPA) axis, stimulation by corticotropin-releasing hormone (CRH), and uterine contraction. CRH plays a major role in
determining the length of gestation. (Behrman & Butler, 2007) (McLean & Smith, 1999) HPA activation and increased expression of CRH lead to progesterone withdrawal and estrogen activation. This leads to cervical ripening, uterine contractility and eventually labor.

In that CRH is secreted from the placenta, abnormalities of placental CRH may be involved in the pathogenesis of preterm labor (McLean & Smith, 1999). Glucocorticoids stimulate production of CRH in the placenta, despite being inhibitors of CRH in the hypothalamus. The lack of nerves in the placenta implicates circulating cytokines as a likely cause of increased CRH production.

**The Oral Cavity**

The oral cavity is populated by over seven hundred species of bacteria (Thomas & Nakaishi, 2006) These bacteria range from beneficial commensals, to opportunistic pathogens, to overt pathogens. Beneficial commensal bacteria, such as *Streptococcus sanguinis* and *Streptococcus gordonii*, can supply nutrients, regulate epithelial development, and contribute to the maturation of the immune system. Beneficial bacteria help to control pathogenic bacteria by competing with them for limited nutrients and space. Opportunistic pathogens, such as *Fusobacterium nucleatum*, may function as commensal bacteria until environmental conditions allow them to overcome host defenses and initiate disease (Handfield et al., 2008).

An overtly pathogenic species of oral bacteria is *Porphyromonas gingivalis*. *P. gingivalis* is a non-motile, Gram-negative, anaerobic, asaccharolytic, coccobacillus found in mature biofilms primarily in the subgingival crevice. *P. gingivalis* is a late colonizer of the oral cavity, attaching to other bacteria such as *Streptococcus oralis, S. sanguinis and S gordonii*. *P. gingivalis* can bind to other late colonizers, such as *F. nucleatum*, which may provide stability and aid in nutrient acquisition (Lamont & Jenkinson, 2000). Study of *P. gingivalis* has revealed an array of virulence factors. Virulence factors of *P. gingivalis* include proteolytic enzymes,
hemagglutinins and fimbriae. *P. gingivalis* produces at least five hemagglutinating molecules (Lamont & Jenkinson, 1998). Hemagglutinins target the cells towards hemin which is a necessary source of iron. Fimbrilin is the monomeric subunit of the major fimbriae and is encoded by the *fimA* gene (Lamont & Jenkinson, 1998). *P. gingivalis* is the major etiologic agent of periodontal disease (Lamont & Jenkinson, 1998)

Periodontal disease is a bacterially induced chronic inflammatory condition consisting of the diseases gingivitis and periodontitis. Gingivitis is reversible inflammation of the gingival tissues around the teeth. Periodontitis results in destruction of the tissues and structures supporting the teeth, including the alveolar bone, periodontal ligament, and connective tissue, eventually leading to the exfoliation of the teeth. It occurs in 5% to 40% of pregnant women (Boggess *et al.*, 2005, Lieff *et al.*, 2004)

**Effects on Pregnancy**

Adverse pregnancy outcomes including preterm birth have been linked to periodontal disease. A review by Boggess and Edelstein concluded that maternal periodontal disease in a risk factor for preterm birth (Boggess & Edelstein, 2006). Pitiphat *et al.* evaluated periodontitis in relation to preterm birth among a cohort of medically insured, middle-class women. The authors concluded that periodontitis was an independent risk factor for adverse pregnancy outcomes among the women studied (Pitiphat *et al.*, 2008).

Animal models have been used to study infection by *P. gingivalis* of pregnant rodents. Lin *et al.* found that *P. gingivalis* infection enhanced fetal growth restriction in mice (Lin *et al.*, 2003). *P. gingivalis* was shown to invade both maternal and fetal tissues by Belanger *et al.* (Belanger *et al.*, 2008). Collins *et al.* examined the effects of a non-disseminating, localized, subcutaneous *P. gingivalis* infection in the golden hamster and concluded that their data suggest
that infection by Gram-negative periodontal pathogens can result in adverse pregnancy outcomes (Collins et al., 1994).

A possible mechanism for periodontal disease to cause preterm birth would be \( P.\ gingivalis \) entering the maternal blood stream and traveling to the placenta where it would induce inflammation. \( P.\ gingivalis \) has been isolated from the blood following scaling and root planning (Lafaurie et al., 2007). \( P.\ gingivalis \) has also been isolated from the human placenta. A study examining periopathogenic microorganisms in placentas of women with preeclampsia found that five of the 16 placental samples from women with preeclampsia and two of the placental samples from healthy control group of women were positive for \( P.\ gingivalis \) with the number of bacterial cells per sample ranging from zero to 3,100 (Barak et al., 2007). Additionally, Leon et al studied women with either preterm premature rupture of the membranes or premature labor and found that eight of 26 study participants had microbial invasion of the amniotic cavity by \( P.\ gingivalis \) as detected by PCR (Leon et al., 2007).

There is epidemiological evidence linking periodontal disease with preterm birth. A review by Xiong et al. concluded that the studies they reviewed suggested that periodontal disease was a risk factor for preterm birth (Xiong et al., 2006). A study published in the Journal of the American Dental Association looked at 1313 pregnant women recruited in Birmingham, Alabama and after adjusting for smoking, race, maternal age, and parity, found an association between the presence of severe or general periodontal disease at 21 to 24 weeks gestation and preterm birth (Jeffcoat et al., 2001).

A 5 year prospective study published in 2001 by Offenbacher et al. reported clinical data from 812 deliveries from a cohort study. After adjusting for race, parity, and baby gender, they concluded that the data provided evidence that maternal periodontal disease and incident
progression are significant contributors to risk for preterm delivery (Offenbacher et al., 2001). Looking at markers of periodontal infection and preterm birth, Jarjoura et al. also concluded that periodontitis is independently associated with preterm birth (Jarjoura et al., 2005).

In 2002 Lopez et al. published the results of a study in which they investigated whether treatment of periodontal disease reduced that risk of preterm low birth weight. There data showed that the women who did not receive treatment for periodontal disease experienced a more than three-fold increase in the risk for preterm birth. (Lopez et al., 2002)

**Experimental Model**

Graham et al. transfected first trimester human trophoblast cells, designated HTR-8/SVneo, with a plasmid containing the gene for the simian virus 40 (SV40) large T antigen (Tag). The result was the HTR-8/SVneo cell line. These cells are phenotypically similar to their parent strain but able to be cultured in vitro for prolonged periods of time. Unlike choriocarcinoma cells, they respond to the migration and invasion signals of TGF-beta in a manner similar to primary cells (Graham et al., 1993). These characteristics make them particularly suitable for studies of placental gene expression which involve cell culture for extended periods of time and thus a good model for this study.
CHAPTER 2
MICROARRAY

Introduction

Specific Aim 1 was to perform microarray analysis on HTR-8/SVneo cells exposed to \textit{P. gingivalis}. Interactions between a pathogen and its host cell are complex and multifaceted. Microarrays are useful in studying these interactions because they allow gene expression to be studied on a global scale (Handfield et al., 2008). Through microarray analysis, genes from pathogens may be identified that are virulence factors involved in pathogenicity (Kato-Maeda et al., 2001) The effect of these virulence genes on the host cell can be studied by monitoring the response of the host cell, such as the differential regulation of various pathways, after infection (Cummings & Relman, 2000).

For this study, microarrays were performed to analyze the effect of \textit{P. gingivalis} on HTR-8/SVneo cells. The effects of \textit{F. nucleatum} were also analyzed to identify which effects were specific to \textit{P. gingivalis}. \textit{F. nucleatum} was chosen as a control because, like \textit{P. gingivalis}, it is a gram-negative anaerobe found in subgingival biofilms. However, unlike \textit{P. gingivalis}, \textit{F. nucleatum} is an opportunistic commensal found in both the presence and absence of disease. It was hypothesized that \textit{P. gingivalis} would alter gene expression in the HTR-8/SVneo cells in a manner that could be hostile to the fetus and thus induce preterm labor.

Methods

HTR-8/SVneo cells were grown to 90% confluency in T-25 flasks in RPMI 1640 media (Sigma) supplemented with 5% fetal bovine serum at 37°C in the presence of 5% CO$_2$. \textit{P. gingivalis} ATCC 33277 and \textit{F. nucleatum} ATCC 25586 were grown anaerobically at 37°C in trypticase-soy broth medium supplemented with yeast extract (1mg/ml), hemin (5ug/ml), and menadione (1ug/ml). Bacteria were harvested in the log phase cells were co-cultured with \textit{P. gingivalis}. 
gingivalis, F. nucleatum, or a control of no bacteria. Co-cultures were carried out in quadruplicate. The cells were lysed with Trizol (Invitrogen, Carlsbad, CA) prior to RNA extraction.

Total RNA was extracted from cells, DNase treated, purified, and quantified. cDNA was synthesized according to a standardized protocol, purified and used as a template for labeled cRNA synthesis. In vitro transcription of cRNA was performed to incorporate biotinylated nucleotides by using a BioArray high-yield RNA transcript labeling kit (Enzo Life Sciences, Farmingdale NY). cRNA was subsequently fragmented and hybridized on Affymetrix HG-U133 Plus 2.0 (Affymetrix). Each sample was studied in parallel, and the samples were not pooled. The microarrays were hybridized for 16 hours at 45°C and then scanned with an Affymetrix GeneChip 3000 scanner.

To begin analysis, an expression filter was applied by removing probe sets that were flagged as absent on all arrays. The signal intensities of the remaining probe sets were ranked according to the coefficient of variation and the 50% of the data with the greatest coefficient of variation was then normalized to a mean of 0 and a standard deviation of 1. Hierarchical cluster analysis was performed on the variance-normalized data set and viewed with GeneCluster and TreeView (Michael Eisen, Stanford University) as shown in Figure 2-1.

Following the initial expression filter, a supervised analysis was performed to investigate differences in gene regulation among experimental conditions using BRB Array Tools (R. Simon and A. Peng-Lam, National Cancer Institute, Rockville, MD). The purpose of this supervised analysis was to identify genes differentially expressed among the treatment classes: cells co-cultured with P. gingivalis, cells co-cultured with F. nucleatum, or cells not exposed to bacteria. The ability of gene identification to predict treatment class was assessed by a leave-one-out
cross-validation using several prediction models including compound covariate predictor, nearest-neighbor predictor, and support vector machine predictor.

Monte Carlo simulations were also used with 2000 permutations of the data set. Differential expression patterns were analyzed with bio-informatics, statistical, and gene ontology tools and databases.

Results

The heat map, Figure 2-1, shows the 2045 genes whose regulation was significantly altered at \( p<0.001 \) among the three classes. The figures in Appendix A show the pathways most affected. The most affected pathways in cells infected with \( P. \text{gingivalis} \) relative to uninfected cells include the cell cycle, MAPK signaling, apoptosis, cell adhesion molecules, cytokine-cytokine receptor signaling, and TGF-beta signaling.

Pathway Express (available at http://vortex.cs.wayne.edu/projects.htm) was used to populate gene ontology trees using genes found to be up or down-regulated by at least 1.25 times relative to their expression in the uninfected control cells at \( p<0.01 \). Genes that were not differentially expressed by a factor of at least 1.25 were filtered out because their inclusion may have lead to falsely high confidence in the effect of microbial challenge on the pathway.

Among the pathways found to be differentially regulated, Pathway Express analysis revealed key factors that were involved in this differential regulation. In the cell cycle and apoptosis pathways these include TNFR and CycD (up-regulated), CycE (down-regulated), and caspase-3/7 and CDK4/6 (not differentially regulated). In the TGF-beta and cell adhesion molecule pathways important factors include that were down-regulated in cells co-cultured with \( P. \text{gingivalis} \) relative to cells not exposed to bacteria include MHC-1, CLDN, OCLN, and CDH1/2. Factors that were up-regulated include Smad2 and Smad3.
Cytokines and factors relating to MAPK that were up-regulated by HTR-8 cells exposed to \textit{P. gingivalis} relative to cells not exposed to bacteria include p38, MKK3, and transcription factor Max of the mitogen-activated kinase pathway as well as cytokines IL-1beta and IL-8 (Figure 2-2). Cytokines IL-1beta and IL-8, as well as MKK3 were also up-regulated in the cells co-cultured with \textit{P. gingivalis} relative to the cells co-cultured with \textit{F. nucleatum} (Figure 2-3). Surprisingly, the macrophage attracting CXCL2 was found to be down-regulated by exposure to \textit{P. gingivalis}. Down-regulation of CXCL2 appears to be in contrast to the up-regulation of pro-inflammatory cytokines, especially chemoattractant IL-8. This down-regulation may be an attempt by \textit{P. gingivalis} to evade host immune responses.

**Discussion**

Study objectives commonly encountered in microarray experiments are “class comparison,” “class prediction,” and “class discovery” (Simon \textit{et al.}, 2003). In class comparison the classes are predefined and they are defined independently of the expression profiles. Class prediction also involves predefined classes but the classes are based on gene expression. Class discovery does not involve predefined classes (Simon \textit{et al.}, 2003).

Analysis can be considered “supervised” if distinctions are made among the samples based on predefined class labels or “unsupervised” if no information about sample grouping is used (Simon, 2003) Cluster analysis, an unsupervised method of analysis, is useful in class discovery. Supervised methods are appropriate for class prediction studies, although over-fitting the predictor can be a major limitation. Over-fitting results from fitting the model to random variations in the data that do not represent true relationships. (Simon \textit{et al.}, 2003)

A class prediction model’s performance can be measured by using a validation set of data that is independent from the training set used to create the model. Cross-validation uses data efficiently as only a small number of specimens are used for the validation set (Simon, 2008). In
leave-one-out cross-validation one specimen is left out at a time and classified by the predictor built by all of the remaining specimens. It is important that all components of class prediction be cross-validated including selection of informative genes, computation of weights for selected informative genes, and creation of a prediction rule. (Simon et al., 2003)

Cross-validation is not sufficient for assessing the significance of a class result. To assess significance, permutation methods can be used to calculate the probability of producing a cross-validated error rate as small as observed given no association between class membership and expression profiles (Simon et al., 2003). A more practical method than examining every possible permutation is with a Monte Carlo method. This involves examining randomly selected permutations and using the proportion that have the same or fewer misclassifications to estimate the achieved significance level (Radmacher et al., 2002).

For the microarrays performed in this study, it was hypothesized that \textit{P. gingivalis} would alter gene expression in the HTR-8/SVneo cells in a manner that could be hostile to the fetus and induce preterm labor. The results of the microarray revealed that exposure of HTR-8/SVneo cells to \textit{P. gingivalis} caused, in addition to the up-regulation of the p38 MAP kinase pathway, an up-regulation of pro-inflammatory cytokines. This finding is significant because inflammation and production of cytokines are known to be associated with preterm birth (Romero et al., 2006, Keelan et al., 2003).

There have been previous studies using microarrays to evaluate cellular host responses to \textit{P. gingivalis}. One such study by Handfield et al. used transcriptional profiling, bioinformatics, statistical and ontology tools to explore genes and pathways modulated upon interaction of \textit{P. gingivalis} or the Gram-negative periodontal pathogen \textit{Aggregatibacter actinomycetemcomitans} with human immortalized gingival keratinocytes. As predicted from the clinical differences in
the bacterial species, transcriptional response in the HIGK cells differed greatly (Handfield et al., 2005).

Serum and glucocorticoid-inducible kinase (SGK) is related to Akt and negatively regulates transcription factor FOXO3A, which participates in apoptosis. Consistent with its anti-apoptotic phenotype, P. gingivalis was found Handfield et al. to activate SGK. Another factor that plays a role in cell survival and proliferation found to be up regulated was cMYC. cMYC can repress transcription of GADD45A, a pro-apoptotic transcription factor (Handfield et al., 2005).

The same study showed that fimbriae-deficient mutant YPF1 had a transcriptional pattern strikingly divergent from the parental strain, indicating a significant role for fimbriae in gene expression. (Handfield et al., 2005). Another study evaluating the role of fimbriae found that human aortic endothelial cells infected with P. gingivalis strain 381 showed up-regulation of 68 genes including IL-8 and IL-6. However in cells infected with the isogenic fimA mutant DPG3 only four of the 68 genes were up-regulated. The authors concluded that fimbriae-mediated invasion up-regulates inflammatory gene expression in human aortic endothelial cells. (Chou et al., 2005)

Milward et al. looked at the effects of P. gingivalis and F. nucleatum on gene expression in oral epithelial cells. They found that F. nucleatum induced a greater number of gene expression changes than did P. gingivalis. They also found many cytokines to be up-regulated which is consistent with the findings of the current study. Cytokines found to be up-regulated after exposure to P. gingivalis in both the Milward study and the current study were IL-8, IL-1alpha, and IL-1beta (Milward et al., 2007).
In another study Zhou and Amar examined gene expression profiles of human macrophages exposed to *P. gingivalis*, or purified LPS or FimA from *P. gingivalis*. They concluded that *P. gingivalis*, as well as its LPS and FimA, induce NF-kB-containing genes through TLR2- or TLR7-MyD88-p38 MAPK pathway (Zhou & Amar, 2007). The current study found that the p38 MAPK pathway is also up-regulated by *P. gingivalis* in the HTR-8/SVneo cells.

A microarray study by Ohno et al. was done in bone marrow derived mouse ST2 cells that showed some pre-osteoblastic characteristics. The study looked at global cellular gene expression profiles in the ST2 cells after exposure to *P. gingivalis*. The investigators concluded that *P. gingivalis* infection does induce gene expression of a wide variety of proinflammatory proteins in stromal cells (Ohno *et al.*, 2006). This was significant to their research because inflammation causes alveolar bone resorption and relates to the current study as well, by showing that *P. gingivalis* promotes inflammation in another cell type. In a subsequent study, the authors investigated the signaling pathways involved in those proinflammatory responses. Unfortunately, p38 MAPK was found to be spontaneously phosphorylated in the unstimulated cells so the effect of *P. gingivalis* infection could not be evaluated (Ohno *et al.*, 2008).

In the current study pathways that were differentially regulated in cells co-cultured in *P. gingivalis* relative to control cells include MAPK signaling, apoptosis, cell cycle, adhesion molecules, and TGF-beta signaling. A recent publication by Inaba et al. found that *P. gingivalis* invasion of HTR-8/SVneo cells leads to G1 arrest and apoptosis. The apoptosis pathway is programmed cell death characterized by nuclear condensation, cell shrinkage, and DNA fragmentation. Caspases are central regulators of apoptosis. Activation of TNFR leads to the activation of caspase-8 and caspase-10, eventually leading to apoptosis. The microarray results in
the present study show an up-regulation of TNFR but no differential regulation of TNF, caspase-3, caspase-7, caspase-9, caspase-10, or Bcl-2. Inaba et al. also found Bcl-2 expression unchanged, however, the authors did find increased expression of caspase 3/7 and caspase 9 after challenge with *P. gingivalis* in a fluorescent substrate assay. (Inaba *et al.*, 2009)

In the cell cycle, the primary G1/S checkpoint controls the transition from the gap phase (G1) to the DNA synthesis phase (S). Inaba et al found that cells challenged with *P. gingivalis* showed decreased levels of cyclin D, CDK4, CDK6, and increased levels of cyclin E for 24 to 72 hours (Inaba et al., 2009). The microarray data from the current study showed HTR-8 cells co-cultured with *P. gingivalis* increased expression of CycD, decreased expression of CycE, and did not differentially express CDK4 or CDK6. Possible reasons for this discrepancy include that Inaba et al. used densiometric analysis of immunoblots while the current study used microarray analysis, and that Inaba et al. used time points of 24, 48, and 72 hours while the array data was collected from cells 2 hours after initial exposure to *P. gingivalis*.

Another group of molecules shown to be affected by *P. gingivalis* in the array data was Cell adhesion molecules (CAMs). Many cell adhesion molecules were down-regulated in the cells exposed to *P. gingivalis* including MHC-1, CLDN, OCLN, and CDH1/2. A possible cause for this would be that the cells were down-regulating these molecules to fight invasion by *P. gingivalis*. Another consideration is that immune molecules are already perturbed in trying to balance destroying pathogens with not rejecting the healthy fetal or maternal tissues.

The transforming growth factor beta (TGF-beta) signaling pathway functions in a wide range of biological systems, playing a role in cell growth, differentiation, and development. Signal initiation involves oligomerization of receptor kinases and phosphorylation of cytoplasmic signaling molecules Smad2 and Smad3. The cells exposed to *P. gingivalis* in the
current microarray study show an up-regulation of TGF-beta receptor type 1, and Smad2/3, but no differential regulation of TGF-beta. Interestingly, p15 is also up-regulated which leads to G1 arrest.

The mitogen-activated protein kinases (MAPK) are involved in many host cell signaling pathways including mitotic response to growth factors, cytokine responses, cytoskeletal reorganization, and stress responses. Factors relating to MAPK that were up-regulated by HTR-8 cells exposed to *P. gingivalis* relative to cells not exposed to bacteria include p38, MKK3, and transcription factor Max (Figure 2-2). A review of the literature indicated the importance of cytokines and inflammation in preterm birth (Goldenberg et al., 2008). Due to its association with cytokine production and inflammation, the p38 pathway of the MAPK pathways was chosen for further analysis later in the study.
Figure 2-1. Divergence of HTR-8/SVneo cells co-cultured with *P. gingivalis*, *F. nucleatum*, or no bacteria. This heat map and was constructed from 2045 probe sets differentially expressed between experimental classes at the significance level of \( P<0.001 \). Probe set signal intensities were variance-normalized, mean-centered across samples, and subjected to hierarchical cluster analysis. Average linkage clustering by un-centered correlation was performed for genes and samples. The degree of similarity between the transcriptional profiles of each sample is expressed by Pearson’s correlation coefficient distance metric which is a measure of dependence obtained by dividing the covariance by the product of the standard deviations, according to the adjacent scale. The expression state of each data point is represented as standard deviations from the mean expression level for that gene in all samples. Red indicates a relative increase, green indicates a relative decrease, and black indicates no relative change of mRNA transcripts for a given genome.
Figure 2-2. Pathway express figure showing the impact of *P. gingivalis* 33277 interaction with HTR-8/SVneo cells, relative to HTR-8/SVneo cells not exposed to bacteria, on the p38 MAPK pathway and cytokine-cytokine receptor interactions. Red terms including p38, MKK3, IL-8 and IL-1beta are transcriptionally up-regulated. The blue term is down-regulated and terms in green were not significantly modulated compared to baseline conditions. Modulated genes were significant at the P<0.01 threshold and were changed by at least a 1.25 fold magnitude.
Figure 2-3. Pathway express figure showing the impact of *P. gingivalis* interaction with HTR-8/SVneo cells relative to HTR-8/SVneo cells co-cultured with *F. nucleatum* upon the p38 MAPK pathway and cytokine-cytokine receptor interactions. Red terms including are transcriptionally up-regulated. The blue term is down-regulated and terms in green were not significantly modulated compared to baseline conditions. Modulated genes were significant at the P<0.01 threshold and were changed by at least a 1.25 fold magnitude.
CHAPTER 3
EFFECOR MOLECULES OF Porphyromonas gingivalis

Introduction

Specific Aim 2 was to identify effector molecules of P. gingivalis. Host-microbe interactions are inherently complex. In the previous chapter, cellular responses of HTR-8/SVneo cells to P. gingivalis were evaluated on a global scale using human DNA microarrays. Study of P. gingivalis has revealed an array of virulence factors including proteolytic enzymes, hemagglutinins and fimbriae. In this chapter an individual virulence factor of P. gingivalis, the major fimbriae, is examined to elucidate its affect on the interaction of P. gingivalis with HTR-8/SVneo cells. Fimbrillin is the monomeric subunit of the major fimbriae and is encoded by the fimA gene (Lamont & Jenkinson, 1998).

The fimA gene is regulated by environmental factors. Expression increases as hemin concentration increases and as temperature decreases from 39 to 34°C (Xie et al., 1997). P. gingivalis appears to express high amount of fimbrillin to facilitate adherence and invasion in the early stages of colonization and repress fimbrillin later to reduce the host immune response. (Xie & Lamont, 1999)

The fimA gene is regulated at both the transcriptional and posttranscriptional level (Xie et al., 2004). At the transcriptional level, a σ-70 like promoter region carries out basal-level transcription while cis-acting regulatory elements are required for maximal transcription of the fimA gene. σ-70 recognized sequences expressed include -10, -35, and UP elements. AT-rich upstream regulator sequences are necessary for full expression of fimA (Xie & Lamont, 1999). Trans-acting components fimbrillin, arginine (Rgp), and lysine (Kgp) bind to the upstream region of the fimA promoter and are necessary for maximal fimA transcription (Xie et al., 2000).
Fimbriae mediate attachment to cells and invasion of previously studied epithelial cells. To examine the effects of fimbriae on *P. gingivalis* infection of HTR-8/SVneo cells a fimbriae-deficient mutant of *P. gingivalis* 33277, designated YPF1, was utilized. YPF1 was created by homologous recombination between a *P. gingivalis* ATCC 33277 chromosomal DNA and a suicide plasmid carrying an internal fragment of the *fimA* gene. The fragment of the *fimA* gene in the suicide plasmid was 0.65-kb in length, cut from restriction sites *PstI* to *HincII*, downstream from the transcriptional regulatory regions and start site (Dickinson *et al.*, 1988, Love *et al.*, 2000). As a result of this insertional inactivation of the *fimA* gene, the YPF1 strain is unable to produce FimA protein and lacks the major fimbriae (Love *et al.*, 2000).

In the current study, two different assays were performed to elucidate the differences between the wild type and *fimA* mutant bacteria when infecting the cells. The first assay, in which formalin fixing was done subsequent to the co-culture, showed the total amount of bacteria including those attached to the cells and those that had invaded the cells. In the second assay, the cells were formalin fixed before co-culture with bacteria to prevent bacterial invasion of the cells. Thus the second assay compared only the ability of the bacteria to attach to the cells.

It was hypothesized that *P. gingivalis* would be able to infect HRT-8/SVneo cells. Furthermore, since fimbriae were necessary for *P. gingivalis* infection of other host cell types, it was expected that the fimbriae-deficient mutant YPF1 would be less effective at infecting HTR-8/SVneo cells.

**Methods**

**Confocal Laser Scanning Microscopy**

HTR-8/SVneo cells, passage 106, were seeded onto glass cover-slips that had been acid-washed and coated with poly-L-lysine. Cells were cultured in RPMI 1640 media (Sigma),
supplemented with 5% fetal bovine serum, at 37°C in 5% carbon dioxide. *P. gingivalis* 33277 and YPF1 *fimA* mutant were grown anaerobically at 37°C in trypticase-soy medium supplemented with yeast extract (1mg/ml), hemin (5ug/ml), and menadione (1ug/ml). Erythromycin (15ug/ml) was added to media for YPF-1. Cells were co-cultured with bacteria harvested in the log phase at a multiplicity of infection of 200 bacteria per cell for 2 hours at 37°C in the presence of 5% CO₂, fixed in 10% formalin for 30 minutes, permeabilized in 0.1% Triton-X 100 for 5 minutes, and blocked in 1% BSA. They were then exposed to anti-33277 antibody from rabbits for 45 minutes, rinsed, and exposed to Alexa-Fluor 488 (Invitrogen) anti-rabbit IgG, Texas Red phalloidin stain (Invitrogen), and DRAQ5 DNA probe.

Images were acquired using a spinning disk confocal system, including a CSU10 Yokagowa confocal scan head, a Roper Cascade II EMCCD 512b camera, a Leica DMIRB microscope, and the open source software package Micro-manager (http://www.micro-manager.org/). Analysis of images was done using Imaris 5.0.1 (Bitplane AG; Zurich, Switzerland) software.

**Fluorescence Assays**

HTR-8/SVneo cells were cultured in RPMI 1640 media (Sigma), supplemented with 5% fetal bovine serum, at 37°C in 5% carbon dioxide. *P. gingivalis* 33277 and YPF1 *fimA* mutant were grown anaerobically at 37°C in trypticase-soy medium supplemented with yeast extract (1mg/ml), hemin (5ug/ml), and menadione (1ug/ml). Erythromycin (15ug/ml) was added to media for YPF-1.

Cells were plated onto black 96-well plates and co-cultured with bacteria harvested in the log phase at a multiplicity of infection of 200 bacteria per cell for 2 hours at 37°C in the presence of 5% CO₂. Cells were then fixed in 10% formalin for 30 minutes, permeabilized with 0.1% Triton-X 100 for 5 minutes, blocked in 1% BSA overnight at 4°C, exposed to anti-33277
antibody, washed, and exposed to Alexa-Fluor 488 anti-rabbit. The procedure was subsequently performed with the formaldehyde fixing prior to infection.

Fluorescence was read by Victor3 Wallac 1420 Multi-label Detection System (Perkin-Elmer). Statistical analysis was done by averaging the results of each well (n=8) in a treatment group and determining the standard deviation. A t-test appropriate for two small samples with different standard deviations was used to verify significance (McClave & Sincich, 2009).

Results

Confocal Laser Scanning Microscopy

Cells were visualized by a confocal laser scanning microscope. Images show that the HTR-8/SVneo cells were infected with *P. gingivalis*. As shown in Figure 3-1, bacterial cells appear to be inside the HTR-8/SVneo cells when view from above. To clarify this, the cells were also viewed from the side. The X-Z projection shown in Figure 3-2 also indicates that *P. gingivalis* were inside the cells. The cells co-cultured with the fimA mutant appear to be infected with fewer bacteria that the cells co-cultured with the wild type bacteria.

Fluorescence Assays

Results from the confocal microscope were confirmed through fluorescence assays. *P. gingivalis* was able to interact with HTR-8/SVneo cells in the 2 hour co-culture. The fimA mutant was far less able to interact with the cells that the wild type bacteria. Infection of HTR-8/SVneo cells in both assays was lower in the cells co-cultured with the fimA mutant than it was in cells co-cultured with wild-type *P. gingivalis*. The assay comparing the difference in total bacterial fluorescence is similar to the assay comparing the difference in fluorescence of bacterial attachment in that both show an approximately 5x decrease in fluorescence between wild type *P. gingivalis* and the fimA mutant.
Discussion

Colonization of the subgingival crevice by *P. gingivalis* requires attachment to antecedent bacteria via fimbriae (Xie et al., 2000). Fimbriae are an important virulence factor of *P. gingivalis*, functioning in both adhesion and invasion. Fimbrilin is the monomeric subunit of the major fimbriae and is encoded by the *fimA* gene (Lamont & Jenkinson, 1998). Regulation of the *fimA* gene is affected by environmental factors such as hemin concentration and temperature (Xie & Lamont, 1999). The fimbriae-deficient mutant YPF-1 was used to elucidate the role *fimA* plays in attachment and invasion of HTR-8/SVneo cells.

It was hypothesized based on the ability of *P. gingivalis* to infect and invade epithelial cells, that *P. gingivalis* would also be able to infect and possible invade HRT-8/SVneo cells. Furthermore, since fimbriae were necessary for *P. gingivalis* infection of other host cell types, it was expected that the fimbriae-deficient mutant YPF1 would be less effective at attaching to, infecting, or invading HTR-8/SVneo cells.

Results from the spinning-disk confocal microscope show that *P. gingivalis* was able to infect HTR-8/SVneo cells. Confocal images appear to show *P. gingivalis* surrounded by cellular actin in both X-Y and X-Z projections. This provides strong, while not entirely conclusive, evidence that *P. gingivalis* were able to successfully invade the HTR-8/SVneo cells. Additional results from the confocal microscope show far fewer *P. gingivalis* present in images of HTR-8/SVneo cells co-cultured with the *fimA* mutant bacteria than in the images of the cells co-cultured with wild type *P. gingivalis*. This leads to the conclusion that the *fimA* mutant was less effective at infecting the HTR-8/SVneo cells.

Florescence assays performed on 96-well plates and read using the Victor3 Wallac 1420 Multi-label Detection System were consistent with the results obtained by the confocal microscope. Furthermore, the florescence assays quantified the difference in the amount of wild
type *P. gingivalis* and *fimA* mutant *P. gingivalis*. A second fluorescence assay was performed comparing infection of live HTR-8/SVneo cells to the infection of HTR-8/SVneo cells fixed in formalin prior to infection. This second assay, Figure 3-6, measures only the ability of the bacteria to attach to the surface of the HTR-8/SVneo cells. If the difference in total interaction shown in the first assay, Figure 3-5, resulted in large part from interaction with the living HTR-8/SVneo cells, then the second assay would not show a similar decrease between the wild type *P. gingivalis* and the *fimA* mutant. The decrease in attachment shown in Figure 3-6 leads to the conclusion that the *fimA* mutant’s decreased ability to interact with HTR-8/SVneo cells is due, either entirely or at least in large part, to its decreased ability to attach to HTR-8/SVneo cells.
Figure 3-1. Confocal image showing *P. gingivalis* after 2 hour co-culture with HTR-8/SVneo cells. *P. gingivalis* bacteria, marked with Alexa-Fluor 488, are shown in green. The actin of HTR-8/SVneo cells, stained with Texas Red phalloidin, is shown in red.

Figure 3-2. Confocal image showing a 20µm slice of the X-Z projection of *P. gingivalis* after 2 hour co-culture with HTR-8/SVneo cells. *P. gingivalis* bacteria, marked with Alexa-Fluor 488, are shown in green. The actin of HTR-8/SVneo cells, stained with Texas Red phalloidin, is shown in red.
Figure 3-3. Confocal image showing \textit{fimA} mutant \textit{P. gingivalis} after 2 hour co-culture with HTR-8/SVneo cells. \textit{P. gingivalis} bacteria, marked with Alexa-Fluor 488, are shown in green. The actin of HTR-8/SVneo cells, stained with Texas Red phalloidin, is shown in red. The cellular nucleus, stained with DRAQ5, is shown in blue.
Figure 3-4. Confocal image shows HTR-8/SVneo cells not exposed to bacteria. The actin of HTR-8/SVneo cells, stained with Texas Red phalloidin, is shown in red.
Figure 3-5. Total fluorescence at 490nm of AlexaFluor labeled *P. gingivalis* after 2 hour infection of HTR-8/SVneo cells. After infection, cells were fixed in formalin and permeabilized with Triton X-100. Asterisks indicate a significant difference (p<0.005) between the indicated cells and cells infected with wild-type *P. gingivalis.*
Figure 3-6. Total fluorescence at 490nm of AlexaFluor labeled *P. gingivalis* attached to HTR-8/SVneo cells after 2 hour co-culture. HTR-8/SVneo cells were fixed with formalin before exposure to bacteria and permeabilized with Triton X-100 after co-culture. Asterisks indicate a significant difference (p<0.025) between the indicated cells and the cells infected with wild-type *P. gingivalis*. 
CHAPTER 4
PATHWAYS

Introduction

Specific Aim 3 was to characterize pathways affected by *P. gingivalis* infection that lead to inflammation. The microarray study showed that several pathways were differentially regulated in cells infected with *P. gingivalis* relative to uninfected controls. Of these pathways the p38 MAPK pathway appeared as a likely candidate to be a pathway that lead to inflammation as a result of *P. gingivalis* infection. The mitogen-activated protein kinases (MAPK) are involved in many host cell signaling pathways including mitotic response to growth factors, cytokine responses, cytoskeletal reorganization, and stress responses. p38 MAP kinase is a serine-threonine kinase activated by phosphorylation of tyrosine and threonine residues by MAP kinase kinase (MEK) 3 and 6, as shown in Fig. 2.2. The p38 pathway leads to cytokine production and inflammation (Schindler *et al.*, 2007). The p38 MAPK pathway has been shown to play a pivotal role in inflammatory cytokine and chemokine gene regulation at both the transcriptional and post-transcriptional levels (Patil & Kirkwood, 2007). Phosphorylation of p38, and the resulting phosphorylation of downstream intermediates, activates gene transcription and stabilizes the AU-rich element (ARE)-containing mRNA (Patil & Kirkwood, 2007).

Transcription factor Max, a basic helix-loop-helix zipper protein that forms a sequence-specific DNA binding complex with Myc, is important for the regulation of cell development and proliferation. Transcription factor Max and the p38 pathway were shown to be up-regulated in the microarray analysis. However, a simple increase in production of mRNA shown in the array is not adequate to show that the p38 pathway is more active in cells infected with *P. gingivalis*. MKK3 and p38 are activated by phosphorylation. Western blots were performed to
determine phosphorylation status of the p38 pathway in cells that had been infected with *P. gingivalis*.

Based on the array data, it was hypothesized that the p38 pathway was up-regulated in cells exposed to *P. gingivalis*. Based on previous experiments evaluating the activity of YPF1, it was expected that the fimbriae-deficient mutant would be less effective at activating the p38 pathway.

**Methods**

**Experimental Conditions**

HTR-8/SVneo cells, passage 126, were grown to 90% confluency in T-25 flasks in RPMI 1640 media (Sigma) supplemented with 5% fetal bovine serum at 37°C in the presence of 5% CO₂. *P. gingivalis* wild type and YPF-1 *fimA* mutant were grown anaerobically at 37°C in trypticase-soy broth medium supplemented with yeast extract (1mg/ml), hemin (5ug/ml), and menadione (1ug/ml). Erythromycin (15ug/ml) was added to media for YPF-1. Bacteria were harvested in the log phase were co-cultured with HTR-8/SVneo cells at a multiplicity of infection of 200 bacteria per cell at 37°C in the presence of 5% CO₂. Time points were 5, 20, and 60 minutes. These shorter periods of co-culture, relative to the co-culture times for the microarray, were chosen because the cellular response being tested was phosphorylation.

**Preparation of Lysates**

After co-culture, cells were washed twice with ice-cold Tris-Buffered Saline (TBS, 20mM Tris-HCl, 150mM NaCl) and lysed in RIPA buffer (50 mM Tris; 150 mM NaCl; 1mM EDTA; 1% Triton-X 100; 5% Protease Inhibitor Cocktail, 20x concentrate from Sigma; 20 mM PMSF; 200 mM Na₃VO₄; 200 mM NaF) for 30 minutes at 4°C. The soluble fraction was collected by centrifugation at 13000 rpm for 10 minutes at 4°C.
Western Blotting

Cell extract was denatured in sample buffer (625 mM Tris-HCl, pH 6.8; 25% glycerol; 2% SDS; 0.01% bromophenol blue; 5% beta-mercaptoethanol, added immediately prior to use), resolved by SDS-PAGE, and electro-transferred to a nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% bovine serum albumin (Santa Cruz) in TBS-T (20mM Tris-HCl, 150mM NaCl, 0.10% Tween 20) for 1 hour at room temperature and then incubated in primary antibody overnight at 4°C. Primary antibodies were from Santa Cruz, diluted 1:200, and were antibodies to either p38, phospho-p38, MEK-3, phospho-MEK3/6, or Max. After washing, the membrane was incubated in biotin-linked bovine anti-rabbit immunoglobulin (Santa Cruz), diluted 1:250, at room temperature for 1 hour. After washing, the membrane was then incubated in streptavidin-peroxidase polymer (SIGMA) diluted 1:10,000 in TBS-T at room temperature for 1 hour. The membrane was then washed five times in TBS-T and once in TBS without Tween-20. Bands were visualized by Pierce ECL Western Blotting Substrate (ThermoScientific, Rockford, IL). The membrane was then stripped in Restore Western Blot Stripping Buffer (ThermoScientific, Rockford, IL), re-blocked in 5% BSA for 1 hour at room temperature, and reprobed with antibodies to β-actin (Cell Signal) overnight at 4°C. Biotin-linked secondary antibody, streptavidin-peroxidase, and visualization by substrate were repeated by the same methods.

Analysis

Blot images were analyzed and quantitated using ImageJ software (written by Wayne Rasband at the U.S. National Institutes of Health http://rsb.info.nih.gov/ij/) following the method outlined at http://www.lukemiller.org/journal/2007/08/quantifying-western-bLOTS-without.html. Values, referred to as areas by ImageJ, obtained from this method represented both the size and the intensity of the bands on the blots. These areas were then standardized with the first band of
each blot, the sample from cells exposed to wild type *P. gingivalis* for 5 min, set equal to a value of 1.0. This resulted in 10 sets of relative values, one each of total p38, phospho-p38, total MEK3, phosho-MEK3, and Max, as well as five blots of β-actin. Each of the five blots was then divided by the values of its corresponding β-actin blot. The data were further analyzed by dividing the β-actin adjusted values of phospho-p38 and phospho-MEK3 by the values of total p38 and total MEK3, respectively.

**Results**

Graphs were generated showing the ratio of total p38 to β-actin (Figure 4-2) and phospho-p38 to actin (Figure 4-3). A third graph (Figure 4-4) was generated showing the ratio of phospho-p38 to total p38. Figure 4-5 shows the ratio of phospho-p38 to total p38 for two sets of blots. At the 20 and 60 minute time points, the values from samples from cells exposed to the wild *P. gingivalis* are significantly higher than the sample from the uninfected control cells (p<0.05).

MEK3 is an activator of p38. Graphs were generated to show total MEK3 adjusted for β-actin (Figure 4-7), phospho-MEK adjusted for β-actin (Figure 4-8), and β-actin adjusted phosphor-MEK3 over β-actin adjusted total MEK3. In one set of blots, all three time points cells infected with wild-type *P. gingivalis* produced a greater ratio of phosphorylated-MEK3 that the uninfected control cells, and cells infected with the *fimA* mutant showed an intermediate value at all three time points (Figure 4-9). However, when results of multiple repetitions of the experiment were looked at together, no real difference among the values could be determined (Figure 4-10).

Max is a transcription factor whose production is up-regulated by phosphorylated p-38. Figure 4-13 shows that cells infected with wild type *P. gingivalis* produced more Max than
uninfected controls, with cells infected with the fimA mutant showing an intermediate value at all three time points. However, none of these differences were statistically significant.

**Discussion**

The effect of *P. gingivalis* on MAPK pathways in HTR-8/SVneo cells has not been thoroughly examined in previous studies; however there have been studies on the effects of *P. gingivalis* on gingival epithelial cells (GECs). GECs frequently encounter *P. gingivalis in vivo*. Although unable to detect p38 MAPK activation in either control or bacterially stimulated cells, results from a study by Watanabe et al suggest the *P. gingivalis* can selectively target components of the MAP kinase pathway (Watanabe *et al.*, 2001). Another study found that infection by *P. gingivalis* accelerates progression of gingival epithelial cells through the cell cycle and that this acceleration was inhibited in fimbriae deficient YPF1 (Kuboniwa *et al.*, 2008).

A study that was done using HTR-8/SVneo cells examined the effects of cadmium on MAPK signaling pathways. The authors concluded that cadmium, as well as hydrogen peroxide, trigger the activation of MAPK signaling pathways in HTR-8/SVneo cells. (Valbonesi *et al.*, 2008). A study focusing on the p38 MAPK pathway in human choriodeciduual explants found that the p38 pathway was involved in causing preterm labor through prostaglandin synthesis in the presence of infection and inflammation (Shoji *et al.*, 2007). In osteoblasts, p38 was found to regulate IL-1beta-stimulated IL-6 expression (Patil *et al.*, 2004).

The current study examined the effects of co-culture with *P. gingivalis* on the p38 MAP kinase pathway in HTR-8/SVneo cells. The p38 MAPK pathway has been shown to play a pivotal role in inflammatory cytokine and chemokine gene regulation at both the transcriptional and post-transcriptional levels (Patil & Kirkwood, 2007). The p38 MAP kinase is a serine-threonine kinase activated by phosphorylation of tyrosine and threonine residues by MAP kinase.
kinase (MEK) 3 and 6. The time points used in these experiments were 5, 20, and 60 minutes. These shorter periods of co-culture were chosen because the cellular response being tested was phosphorylation.

Based on the array data, it was hypothesized that the p38 pathway was up-regulated in cells exposed to \textit{P. gingivalis}. Results of this study showed that co-culture with \textit{P. gingivalis} did increase the phosphorylation of p38 and of MEK3. Additionally, co-culture with the fimbriae-deficient mutant YPF1 resulted in a diminished effect on phosphorylation of p38 and MEK3. However, the only results that were statistically significant at any reasonable p-value were the differences in p38 phosphorylation relative to total p38 between cell co-cultured with wild type \textit{P. gingivalis} and those cells not exposed to any bacteria at 5 and 20 minutes (Figure 4-5).

Therefore, these results cannot be said to definitively confirm the hypothesis that the pathway of p38 MAP kinase is activated by infection with \textit{P. gingivalis}, and to a lesser extent, with infection by the \textit{fimA} mutant in HTR-8/SVneo cells. However, the results do indicate that this is a possibility and may be shown to be true with further study. This finding would be significant because the p38 pathway leads to the production of cytokines that have also been associated with preterm birth (Patil & Kirkwood, 2007).
Figure 4-1. Western immunoblots of HTR-8/SVneo cells infected with wild type *P. gingivalis*, *fimA* mutant *P. gingivalis*, and uninfected controls for 5, 20, or 60 minutes. The blots were probed with antibodies to p38 and phospho-p38 and then stripped and reprobed with antibodies to β-actin.

Figure 4-2. Results of densitometric analyses of the ratio of total p38 band intensity to β-actin band intensity.
Figure 4-3. Results of densitometric analyses of the ratio of phospho-p38 band intensity to β-actin band intensity.

Figure 4-4. Results of densitometric analyses of the ratio of phospho-p38 band intensity to total p38 band intensity.
Figure 4-5. Mean (± the standard deviation) of phosphorylated p38 over total p38 for two sets of blots. Asterisks indicate a significant difference (p<0.05) between cells infected with wild type *P. gingivalis* and uninfected control cells.
Figure 4-6. Western immunoblots of HTR-8/SVneo cells infected with wild type *P. gingivalis*, *fimA* mutant *P. gingivalis*, and uninfected controls for 5, 20, or 60 minutes. The blots were probed with antibodies to total MEK3 and phospho-MEK3 and then stripped and reprobed with antibodies to β-actin.

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Figure 4-7. Graph showing the results of densitometric analyses of the ratio of total MEK3 band intensity to β-actin band intensity.
Figure 4-8. Graph showing the results of densitometric analyses of the ratio of phospho-MEK3 band intensity to β-actin band intensity.

Figure 4-9. Graph showing the results of densitometric analyses of the ratio of phospho-MEK3 band intensity to total MEK3 band intensity.
Figure 4-10. Mean (± the standard deviation) of phosphorylated MEK3 over total MEK3 for two sets of blots.
Figure 4-11. Western immunoblots of HTR-8/SVneo cells infected with wild type *P. gingivalis*, *fimA* mutant *P. gingivalis*, and uninfected controls for 5, 20, or 60 minutes. The blots were probed with antibodies to transcription factor Max and then stripped and reprobed with antibodies to β-actin.

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<tr>
<th>Lane</th>
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<td><em>P. gingivalis</em> for 5min</td>
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<td>FimA mutant for 5min</td>
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<td><em>P. gingivalis</em> for 20min</td>
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<td>5</td>
<td>FimA mutant for 20min</td>
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<td>6</td>
<td>No bacteria for 20min</td>
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<td>7</td>
<td><em>P. gingivalis</em> for 60min</td>
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<td>8</td>
<td>FimA mutant for 60min</td>
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<td>9</td>
<td>No bacteria for 60min</td>
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Figure 4-12. Results of densitometric analyses of the ratio of Max band intensity to β-actin band intensity.
Figure 4-13. Mean (± the standard error) of transcription factor Max for three sets of blots.
CHAPTER 5
CYTOKINES

Introduction

Cytokines are small proteins that function in cellular signaling. Cytokines have been found to be related to preterm birth. Figueroa et al found that concentrations of IL-1alpha, IL-6, and IL-8 were significantly higher in the amniotic fluid of women who delivered within seven days of the amniocentesis at which their samples were taken than those that delivered more than seven days later (Figueroa et al., 2005).

IL-1 can be found in elevated concentrations in the amniotic fluid of women experiencing preterm labor. IL-1beta was found to be the key subtype (Hill, 2000) and the elevated IL-1 activity in fluid with infection was more consistent with IL-1beta than IL-1alpha (Hill, 2000, Mitchell et al., 1993). Steinborn et al found increased IL-1beta production in women delivering preterm compared to women delivering at term and not in labor (Steinborn et al., 1996). Animal studies also indicate IL-1beta as a trigger of preterm birth. Romero et al showed that IL-1 stimulated preterm delivery in mice (Romero et al., 1991). A study by Sadowsky et al found that intra-amniotic infusions of IL-1beta in pregnant rhesus monkeys induced preterm labor in all cases (n = 5) (Sadowsky et al., 2006).

IL-6 is a multipotent pro-inflammatory cytokine that functions in B cell stimulation as well as promoting inflammation. Human trophoblasts are known to produce IL-6 (Saji et al., 2000). IL-1 and IL-6 have been shown to stimulate CRH from human placental trophoblasts in vitro (Petraglia et al., 1990). IL-6 has been found to be elevated in the amniotic fluid of women within seven days of delivery (Figueroa et al., 2005).

Women experiencing preterm labor also have elevated amniotic fluid and serum concentrations of chemokines. Chemokines are chemoattractant cytokines that induce
chemotaxis in nearby cells by setting up a concentration gradient leading neutrophils to the site of infection. IL-8 was the first chemokine to be characterized. IL-8 has been found to be elevated in the amniotic fluid of women experiencing preterm labor (Hill, 2000) Dowd et al also found that IL-8 levels in the cervical secretions of women who deliver preterm were significantly higher than those who did not deliver preterm (Dowd et al., 2001).

Based on the array data, it was hypothesized that production of cytokines IL-8 and IL-1beta would increased in cells infected with \textit{P. gingivalis}. Previous experiments conducted using YPF1 and the HTR-8/SVneo cells lead to the prediction that YPF1 would be less effective at increasing cytokine production.

The microarray data indicated that cytokines IL-8 and IL-1 were upregulated in HTR-8/SVneo cells infected with \textit{P. gingivalis}. However, cytokines can be regulated post-transcriptionally as well as at the mRNA level. ELISAs were performed to confirm increased production of cytokines. The use of ELISA also allowed for additional cytokines and multiple time points to be evaluated.

**Methods**

HTR-8/SVneo cells were grown to 90% confluency in T-25 flasks in RPMI 1640 media (Sigma), supplemented with 5% fetal bovine serum, at 37°C Celsius in 5% carbon dioxide. \textit{P. gingivalis} 33277 and YPF1 \textit{fimA} mutant were grown anaerobically at 37°C in trypticase-soy medium supplemented with yeast extract (1mg/ml), hemin (5ug/ml), and menadione (1ug/ml). Erythromycin (15ug/ml) was added to media for YPF-1.

Cells were co-cultured with bacteria harvested in the log phase at a multiplicity of infection of 200 bacteria per cell for 2 hours at 37°C in the presence of 5% CO₂. After 2 hours the supernatant from 2 hour flasks was collected and cell lysis buffer (Sigma) was then used to solubilize cells. Supernatant and lysate samples were frozen at -20°C until use. The remaining
flasks were washed at 2 hours with PBS and given fresh media. Samples for 6 hour and 24 hour
time points were collected in the same manner as the 2 hour time point.

Enzyme-linked immunosorbent assay (ELISA) was used to quantify production of
cytokines IL-8, IL-6, and IL-1beta. Cell culture supernatant was measured for IL-6 and IL-8 and
cell lysates were measured for IL-1beta. Commercially available reagents and kits (R&D
Systems) were used for ELISAs in accordance with the manufacturer’s instructions.

Results

The results of the ELISA measuring the HTR-8/SVneo cell lysates, Figure 5-1, show the
measured concentration of IL1beta. There is a significant increase in the concentration of IL-
1beta in cells co-cultured with wild type P. gingivalis relative to the cells not exposed to bacteria
(p>0.005) at all three time points, which include 2, 6, and 24 hours. There is also a significant
decrease (p>0.005) in the concentration of IL-1beta in lysates from cells exposed to the fimA
mutant P. gingivalis relative to the wild-type P. gingivalis at all three time points. Surprisingly,
at the 24 hour time point the concentration of IL-1beta is lower in cells exposed to the fimA
mutant than in cells exposed to no bacteria.

ELISA analysis of the cell supernatant also showed an increase in cytokine production
after exposure to P. gingivalis. Figure 5-2 shows the concentration of IL-6 was significantly
lower (p<0.0005) in the uninfected control cell supernatant than in the supernatant from cells
infected with wild type P. gingivalis at 6 and 24 hours. At 24 hours, the supernatant from cells
infected with the fimA mutant P. gingivalis had a significantly lower (p<0.0005) concentration of
IL-6 than the cells infected with wild type P. gingivalis and a significantly higher (p<0.0005)
concentration than cells no exposed to bacteria.

The concentration of IL-8, as shown in Figure 5-3, was significantly lower in the
supernatants from cells not exposed to bacteria than those of the cells co-cultured with the wild
type \textit{P. gingivalis} at 6 hours (p<0.001) and 24 hours (p<0.005). At 24 hours, the concentration of IL-8 in the supernatant from the cells co-cultured with the \textit{fimA} mutant was significantly lower than the wild type (p<0.001) and slightly, but significantly (p<0.01), higher than the concentration from cells not exposed to bacteria.

**Discussion**

The effects on cytokine production in trophoblast cells of various species of pathogenic bacteria have been studied. Griesinger et al studied the effects of \textit{Escherichia coli}, \textit{Bacteroides fragilis}, \textit{Mycoplasma hominis}, \textit{Staphylococcus aureus}, and \textit{Streptococcus agalactiae}, all of which have been identified in intrauterine infections. They found that these pathogenic microorganisms induced a dose- and time-dependent release of IL-1beta, IL-6, and IL-8 (Griesinger, Saleh et al. 2001).

The hypothesis being tested in this study was that infection of HTR-8/SVneo cells with \textit{P. gingivalis} would increase production of cytokines IL-8 and IL-1beta. Results of ELISA confirmed the up-regulation of IL-1beta and IL-8 at the two hour time point when the array samples were collected. However, the use of ELISA allowed for the inclusion of additional time points. The increase in cytokine production was much greater at 24 hours than 2 hours in for both cytokines. Interestingly, the finding that \textit{P. gingivalis} infection results in increased production of IL-8 is in contrast to previous studies that have found \textit{P. gingivalis} inhibits production of IL-8 by human gingival epithelial cells (Darveau \textit{et al.}, 1998).

Additional cytokines, TNF-\(\alpha\) and IL-6, were also evaluated using ELISA. TNF-\(\alpha\) was not produced in a measurable concentration by any of the cells at any time point. IL-6, however, was shown to be increased in the 6 and 24 hour time points. In addition to the cells not exposed to bacteria and the cells exposed to wild type \textit{P. gingivalis}, cytokine concentrations produced from cells infected with YPF1 were measured and found to be lower than cell infected with wild
type *P. gingivalis*. These results are important because the cytokines IL-1beta, IL-6, and IL-8 have all been attributed to preterm birth.
Figure 5-1. Graph depicting measured concentration of IL-1beta present in cell culture supernate. The horizontal axis shows time, in hours, following initial exposure to bacteria. The vertical axis shows the concentration, in pg/ml, of IL-1beta. The blue bars show data from cells infected with wild type *P. gingivalis* ATCC 33277. The red bars show data from cells infected with a *fimA* mutant. The green bars show data from cells that were not exposed to bacteria. Asterisks indicate a significant difference (p<0.005) between the indicated cells and the cells infected with wild-type *P. gingivalis* at the same time point.
Figure 5-2. Graph depicting measured concentration of IL-6 present in cell culture supernatant. The horizontal axis shows time, in hours, following initial exposure to bacteria. The vertical axis shows the concentration, in pg/ml, of IL-6. The blue bars show data from cells infected with wild type *P. gingivalis* ATCC 33277. The red bars show data from cells infected with a *fimA* mutant. The green bars show data from cells that were not exposed to bacteria. Asterisks indicate a significant difference (p<0.0005) between the indicated cells and the cells infected with wild-type *P. gingivalis* at the same time point.
Figure 5-3. Graph depicting measured concentration of IL-8 present in HTR-8/SVNEOcell culture supernatant. The horizontal axis shows time, in hours, following initial exposure to bacteria. The vertical axis shows the concentration, in pg/ml, of IL-8. The blue bars show data from cells infected with wild type *P. gingivalis* ATCC 33277. The red bars show data from cells infected with a *fimA* mutant. The green bars show data from cells that were not exposed to bacteria. Asterisks indicate a significant difference (\( **p<0.001; *p<0.005 \)) between the indicated cells and the cells infected with wild-type *P. gingivalis* at the same time point.
Preterm birth, the leading cause of infant mortality, has increased in the United States over the past 20 years (Michalowicz & Durand, 2007). Maternal periodontal disease is associated with preterm birth but the pathogenesis is not fully understood. The mechanism proposed in this study was *P. gingivalis* traveling from the mouth to the placenta via the mother’s blood stream induced inflammation in the fetal tissues. This is reasonable to suggest because *P. gingivalis* and other oral bacteria have been found in fetal tissues (Barak et al., 2007).

This study examined the effects *P. gingivalis* ATCC 33277 has on HTR-8/SVneo human placental cells *in vitro*. Cells were exposed to bacteria at a multiplicity of infection of 200 at 37°C in the presence of 5% CO₂. The effects of bacterial exposure on cells were measured by microarray, confocal laser scanning microscopy, fluorescence assay, western blot, and ELISA.

The results of the microarray revealed that exposure of HTR-8/SVneo cells to *P. gingivalis* caused the differential regulation of the p38 MAP kinase pathway and pro-inflammatory cytokines. This finding is significant because inflammation is known to be associated with preterm birth (Romero *et al.*, 1998)

Images from the confocal laser scanning microscope show that *P. gingivalis* was able to infect HTR-8/SVneo cells. The fimbriae-deficient mutant YPF1 was less effective at infecting HTR-8/SVneo cells. This indicates that the major fimbriae function in infection of the HTR-8/SVneo cells. Fluorescence assays comparing infection of live HTR-8/SVneo cells to HTR-8/SVneo cells fixed in formalin prior to infection showed similar results. This indicates that the YPF1 strain was less able to attach to the HTR-8/SVneo cells, thus implying a role for fimbriae in attachment to HTR-8/SVneo cells.
Western blots were performed to study the effect of *P. gingivalis* infection on the p38 MAP kinase pathway in HTR-8/SVneo cells. The p38 MAP kinase is a serine-threonine kinase activated by phosphorylation of tyrosine and threonine residues by MAP kinase kinase (MEK) 3 and 6. Based on the array data, it was hypothesized that the p38 pathway was upregulated in cells exposed to *P. gingivalis*. Results of this study showed that infection with *P. gingivalis* increased the phosphorylation of p38 and of MKK3. Additionally, infection with the fimbriae-deficient mutant YPF1 resulted in a diminished effect on phosphorylation of p38 and MKK3. These results confirm the hypothesis that the pro-inflammatory pathway of p38 MAP kinase is activated by infection with *P. gingivalis*, and to a lesser extent, with infection by YPF1. This finding is significant because the p38 MAPK pathway has been shown to play a pivotal role in inflammatory cytokine and chemokine gene regulation at both the transcriptional and post-transcriptional levels (Patil & Kirkwood, 2007).

ELISA was used to further test the hypothesis that infection of HTR-8/SVneo cells with *P. gingivalis* would increase production of specific cytokines. Results of ELISA confirmed the up-regulation of IL-1beta and IL-8 at the two hour time point when the array samples were collected. Additionally, the increase in cytokine production by cells infected with *P. gingivalis* relative to uninfected controls was much greater at 24 hours than 2 hours for both cytokines.

Additional cytokines, TNF-α and IL-6, were also evaluated using ELISA. TNF-α was not produced in a measurable concentration by any of the cells at any time point. IL-6, however, was shown to be increased in the 6 and 24 hour time points. In addition to the cells not exposed to bacteria and the cells exposed to wild type *P. gingivalis*, cytokine concentrations produced from cells infected with YPF1 were measured and determined to be lower than that of the cells.
infected with wild-type *P. gingivalis*. These results are important because the cytokines IL-1beta, IL-6, and IL-8 have all been attributed to preterm birth.

Taken together, the results of this study demonstrate that *P. gingivalis*, found in previous studies to be present in some placentas, has the ability to induce an inflammatory response in cultured human placental cells. Given the role that inflammation is understood to play in preterm parturition, this suggests a role for *P. gingivalis* in the cause of preterm birth.

The results of this study suggest that benefits may be obtained from further study. Additional studies may involve other clinical models such as animal models or the use of primary cells *in vitro*. Various methods may also be used to enhance understanding of the HTR-8/SVneo pathways *P. gingivalis* affects. For example, study into what HTR-8/SVneo cell-surface receptors are used by *P. gingivalis*. Of particular interest, the role of the TGF-beta pathway in leading to G1 arrest in the cell cycle or to the p38 MAPK pathway. In the future, more research may be done to further elucidate the role *P. gingivalis* plays in preterm birth.
### APPENDIX

<table>
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**Figure A-1.** Pathways most affected by co-culture with *P. gingivalis* relative to the uninfected control (p<0.001).
Figure A-2. Pathways most affected by co-culture with *P. gingivalis* relative to cells co-cultured with *F. nucleatum* (p<0.001).
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Sally Riewe was raised in Satellite Beach, Florida. She attended Surfside Elementary, DeLaura Jr. High, and Satellite High School. She received a Bachelor of Science degree in environmental sciences from the University of Florida in 2006 and a Masters of Science degree in medical science from the University of Florida in 2009. She hopes to attend dental school and continue her education to become a dentist. She would like to practice and research dentistry in the state of Florida. She currently lives with her fiancé Bret, dog Kallie, cat Lynx, and some birds and fish. She enjoys gardening in the Florida sunshine.